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Functional analysis of RET in MEN2

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Dedicado a todos mis seres amados, a aquellos que estan, y a los que ya se fueron...

A Patricia, por iluminar mi camino,
por compartir sueños e ilusiones,…
un amor y una vida.

INDEX

SCOPE

Cancer results from genomic alterations leading to uncontrolled proliferation of a given population of cells. These alterations mainly occur in genes that encode proteins whose function is to maintain the stability of the genome and/or control cell proliferation. The best-known cancer-related genes are tumour-suppressor genes, DNA repair genes and oncogenes, respectively. Tumour-suppressor genes encode proteins that have as a normal function to control or inhibit cell division. The mutant allele behaves in a recessive way i.e. as long as the cell contains one normal allele, there will be tumour suppression activity. When both alleles are lost, tumour suppression will be abrogated. DNA repair genes encode proteins that are involved in genomic stabilisation. Inactivation of repair genes results in genomic destabilisation, giving rise to an accumulation of mutations and enhances the probability of mutations in tumour-suppressor genes and oncogenes, respectively. Repair genes mostly behave, as tumour suppressor genes, in a recessive way. Oncogenes encode proteins that promote cell proliferation, migration and differentiation. They have a dominant action: one mutant allele may transform a normal cell into a tumour cell.

Within the latter group, receptor tyrosine kinases (RTKs) have appeared during the last twenty years as major players in the hallmarks of cancer. They are a sub-class of the cell-surface growth factor receptors with intrinsic ligand-controlled tyrosine kinase activity. Normally, they regulate crucial biological processes in the cell, but when mutated, RTKs can become active independent of a ligand or may by altered signalling properties become oncoproteins. Understanding the molecular bases of cancer will not only help us to get better insights in genotype-phenotype correlations, it might also help in designing new therapeutic strategies.

My doctoral thesis focuses on RET, a tyrosine kinase receptor expressed in neural crest-derived tissues and associated, when mutated, with different inherited neural crest disorders, namely Multiple Endocrine Neoplasia type 2 (MEN2) and Hirschprung disease. The aim of this study was to determine the signalling profiles of different RET mutations as compared to wild-type RET signalling. We wanted to understand how the different signalling profiles contribute to the different disease phenotypes. Furthermore, as no effective systemic treatment is available for MEN2, we evaluated imatinib, a tyrosine kinase inhibitor as a candidate for that purpose.

This work shows that RET receptor signalling is a fascinating model to understand how genetic alteration within a single gene can give rise to different inherited human disorders by altering the functional (signalling) properties and expression of the encoded protein.

Chapter 1
Current concepts in RET-related genetics and signalling

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A modified version of this chapter has been submitted as a review

INTRODUCTION

The *RET* (REarranged during Transfection) gene, discovered in 1985 when NIH-3T3 cells were transformed by transfection with human lymphoma DNA (1), is localized at 10q11.2 and contains 21 exons. By alternative splicing, three different isoforms of the RET protein are generated, containing 51 (RET51), 43 (RET43) and 9 (RET9) amino acids in the COOH terminal tail, respectively (2). RET51 and RET9 are the most prevalent isoforms *in vivo*. The RET51 isoform shows the highest transforming activity and kinase activity in *in vitro* assays (3). Several observations suggested that the different isoforms have different tissue-specific effects during embryogenesis (4). RET is the receptor for members of the glial cell line-derived neurotrophic factor (GDNF) family of ligands (GFL): GDNF, Neurturin (NRTN), Persephin (PSPN) and Artemin (ARTN) (5). However, to stimulate RET, these ligands need to form a complex with glycosylphosphatidylinositol (GPI)-anchored co-receptors, called GDNF family receptors alpha (GFRα) (6). Four such co-receptors have been identified: GFRα1, GFRα2, GFRα3 and GFRα4 (7). Analysis of mice lacking the genes of GFLs and their related receptors, has confirmed the importance of these factors in neural (crest) development (7).

During vertebrate embryogenesis, RET is expressed in the developing excretory system, in all lineages of the peripheral nervous system (PNS), and in motor and catecholaminergic neurons of the central nervous system (CNS) (8). RET is also expressed in tumors of neural crest origin, such as medullary thyroid carcinomas (MTCs), pheochromocytomas and neuroblastomas (9). Whether the three isoforms play different roles in tumorigenesis is still unknown.

The role of *RET* in human disorders was first described when somatic rearrangements of *RET* (named *RET/PTC*) were found in papillary thyroid carcinomas (PTCs) (10). The proteins encoded by the *RET/PTC* oncogenes possess constitutive tyrosine kinase activity. These RET/PTC onco-proteins -of which over 10 have been described- are all chimeras proteins which consist of the NH2 terminal region of different proteins fused to the catalytic domain of RET (11). The clinical relevance of *RET* became even more apparent when germline *RET* mutations, giving rise to constitutively activated RET proteins were discovered as the cause of the cancer syndrome Multiple Endocrine Neoplasia type 2 (MEN2) (12). On the other hand, *RET* mutations that result in a loss of RET function were found to be

associated with Hirschsprung's disease (HSCR); a developmental disorder characterized by the absence of enteric ganglia cells in the intestinal tract (13).

This review focuses on current concepts in genetics and molecular mechanisms underlying the different inherited human neural crest disorders in which RET dysfunction plays an essential role.

RET STRUCTURE

RET is a tyrosine kinase receptor which belongs to the Platelet-Derived Growth Factor receptor subfamily. Like other receptor tyrosine kinases RET contains an extracellular ligand binding domain, a single transmembrane domain and an intracellular tyrosine kinase domain (14) (Fig. 1). The extracellular region of the RET molecule consists of a domain with similarity to the cadherin family of Ca^{2+}-dependent cell adhesion molecules (14) and a cysteine-rich domain located close to the plasma membrane (12). These cysteines, which are highly conserved in RET receptors from various species, play a critical role in the formation of intramolecular disulfide bonds to shape the tertiary structure of the RET protein (12). The intracellular part of RET contains a tyrosine kinase domain with the conserved features of tyrosine kinase proteins (Fig.1).

Figure 1

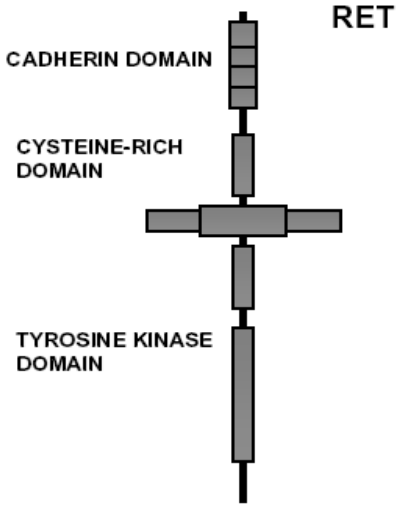

Figure 1

RET and its functional domains

WILD TYPE RET ACTIVATION AND SIGNALLING

RET signalling plays a crucial role in the development of the enteric nervous system (ENS) in kidney organogenesis and in spermatogenesis (16). Activation of the tyrosine kinase domain of RET occurs through transient dimerization induced by the formation of a macromolecular GFL/GFRα/RET complex (Fig. 2). Although the exact mechanism of RET activation is not yet entirely clear, the involvement of lipid rafts coupled to the formation of the GFL/GFRα/RET complex has generally been accepted as reviewed by Manie et al (17).

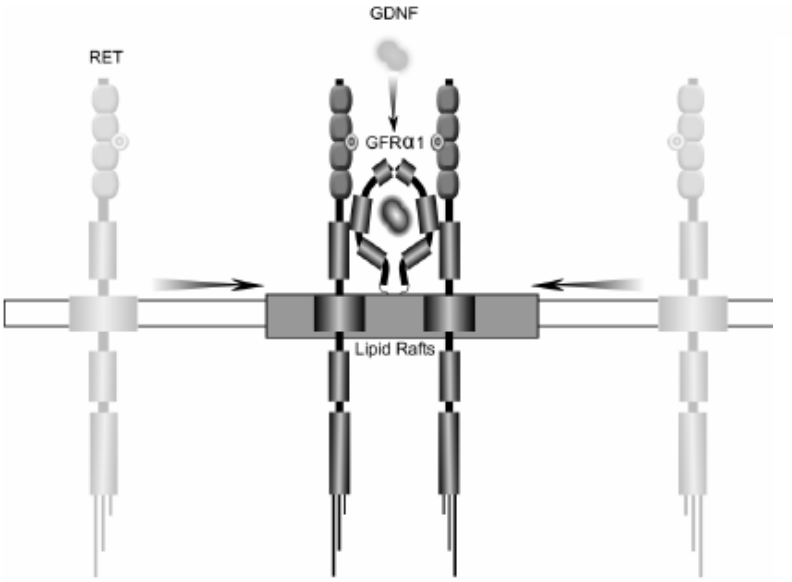

Figure 2
Activation of wild type RET
Ligand regulated activation of wild type RET takes places after its ligand (GDNF) binds to the GPI-anchor co-receptor (GFR-α1). After GDNF/GFRα1 complex formation, RET is recruited to rafts where the GDNF/GFR-α1 protein complex is present and transient dimerization takes place.

RET wild type dimerization and activation results in trans-phosphorylation of several intracellular tyrosine residues, which serve as docking sites for adaptor proteins and results in the activation of various signalling pathways. Phosphotyrosine residues 905, 981, 1015, 1062 and 1096 serve as docking sites for GRB7/10, SRC, PLCγ, SHC/ENIGMA/FRS2/IRS1-2/DOK4-5 and GBR2, respectively (18, Fig. 3). In general, the signalling pathways activated by wild type RET include RAS-MAPKs, PI3K, c-Jun N-terminal Kinase (JNK), p38, SRC, ERK-5 and PLC-γ (18, 19, Fig. 3).

RET tyrosine 1062 plays an important role in the histogenesis of the enteric nervous system and in nephrogenesis. Mutation of tyrosine 1062 in RET causes a marked decrease of enteric neurons and renal hypoplasia in a mouse model (20). Wild type RET interaction with SRC occurs via tyrosine 981 and this interaction promotes neuronal survival (19). Phosphotyrosine 905 is interacting with GRB7/10 docking proteins upon RET activation. The signalling

pathways downstream of GRB7/10, as well as their biological relevance, are not yet clear (21). Phosphotyrosine 1015 binds and activates PLC-γ (4). PLC-γ hydrolyzes PtdIns (4, 5) P2, into inositol 1, 4, 5 trisphosphate (IP3) and diacylglycerol (DAG). IP3 promotes the release of Ca^{2+} from intracellular stores, which results in activation of many enzymes, such as Ca^{2+} regulated isoforms of protein kinase C and Ca^{2+} calmodulin-regulated protein kinases. DAG is known to stimulate DAG-regulated protein kinase C isoforms. Interestingly, PKCα binds to RET and in turn causes down-regulation of its kinase activity and downstream signalling, thus functioning as a negative feedback loop to modulate RET activity (22). Furthermore, it has been demonstrated that NRTN (but not GDNF) stimulates neurite outgrowth and that this could be blocked by U73122, a selective PLCγ inhibitor, suggesting a role of the PLCγ pathway, which is initiated via tyrosine 1015, in RET-mediated neurite growth and differentiation (23). Tyrosine 1096 is only present in the long isoform of RET and serves when phosphorylated as a docking site for GRB2, which activates the SOS/RAS/ERK and PI3K pathways, respectively (24).

In addition, RET can activate members of the RHO family of GTPases, including RHO, RAC and CDC42, that are involved in the reorganization of the cytoskeleton responsible for cell motility and morphology (25). The biological importance of RET-mediated activation of P38, JNK, ERK5 and PKCα still needs to be elucidated.

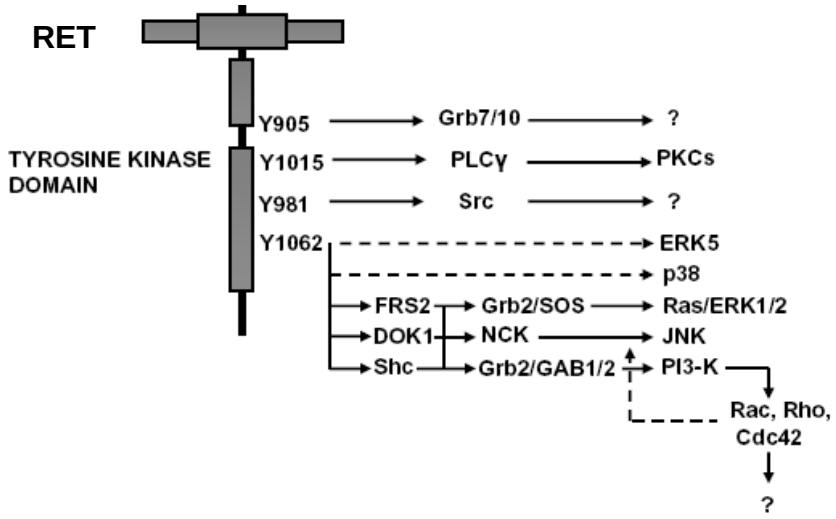

Figure 3

Scheme of the main signaling pathways activated by wild type RET.

Arrows indicate direct interactions, whereas dashed arrows indicate indirect interactions

RET INDEPENDENT GDNF/GFRα SIGNALLING

Activation of wild type RET occurs via a GFL/GFRα/RET macromolecular complex. It has been shown that GFRαs can also signal independently of RET. Activation of GFRα1, upon stimulation by GDNF, activates the RAS/MAPK, PLC-γ and CREB pathways and induces FOS in RET-deficient cell lines and primary neurons, via SRC family kinases (26). MET, a close family member of RET, possibly contributes to this RET-independent GDNF signalling as GDNF induces MET phosphorylation in RET-deficient but GFRα1-positive cells and in RET/GFRα1-coexpressing cell lines as well. GDNF-induced MET phosphorylation was mediated by Src family kinases (27). Furthermore, neural cell adhesion molecule (NCAM) has been shown to mediate RET-independent GFL signalling in neuronal cells (28). If and how RET-independent GDNF/GFRα signaling is of importance for the development of RET-related diseases is yet unclear. RET-independent signalling by GDNF is reviewed in detail by Sariola et al (29).

The signalling networks activated by GFLs are much more complex than initially thought, involving not only different co-receptors but also alternative receptors for GDNF family members as well as cross-talk between RET and other receptor tyrosine kinases.

NEURAL CREST DISORDERS ASSOCIATED WITH RET MUTATIONS

Multiple Endocrine Neoplasia type 2

Germline missense mutations resulting in activation of the RET protein cause MEN2, a dominantly inherited cancer syndrome affecting neuroendocrine organs (12). Depending on the affected tissues and mutations found, three different clinical subtypes can be distinguished:

a) MEN2A characterized by medullary thyroid carcinoma (MTC), a tumor of the parafollicular c-cells that are responsible for the secretion of calcitonin by the thyroid gland, pheochromocytoma (PC), a tumor affecting the adrenal chromaffin cells and hyperparathyroidism (HPT) (30). MTC occurs in all MEN2A patients, PC is observed in approximately 50% of the cases and HPT in 15%-30% of the patients.

b) MEN2B where patients also develop MTC (100%) and PC (approximately 50%), but instead of HPT these patients develop a more complex clinical phenotype including

ganglioneuromas in the tongue, lips and eyelids, intestinal ganglioneuromas, thickened corneal nerves as well as a marfanoid habitus. It is considered to be the most aggressive subtype of MEN2 with the earliest age of onset (31).

c) Familial MTC (FMTC) is characterized by the manifestation of MTC in four or more members of an affected family. As tumors are only found in the thyroid, FMTC should strictly speaking not be called a MEN2 subtype. FMTC is considered to be the least aggressive MEN2 phenotype (33).

Figure 4 schematically shows the identified genotype-phenotype correlations and a subdivision of *RET* mutations according to the position in the coding sequence. The functional consequences are given in Table 1 (12, 33, 34, 35). A recent update of all discovered MEN2- and FMTC-associated *RET* mutations is provided by Arighi (16). According to genotype-phenotype studies and from analysis of RET-MEN2 mutations in cellular systems and mice models, aggressiveness of MTC (which has a nearly 100% penetrance in MEN2 and FMTC) seems to correlate with the genotype (Table 1). An overview of these functional studies and their results is presented in Table 2.

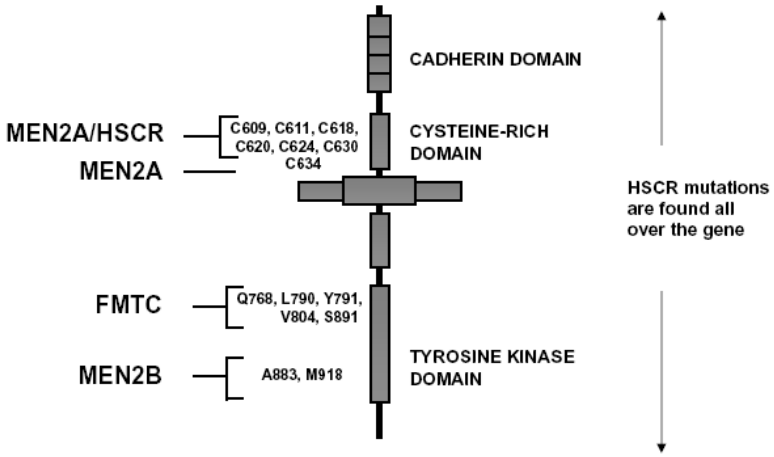

Figure 4

Genotype-phenotype correlation of specific disease phenotype-associated RET mutations in MEN2.

RET polymorphisms and haplotypes in RET associated endocrine tumors

Not only highly penetrant germline *RET* mutations play a major role in cancer development. There has been a large number of reports suggesting that low penetrant *RET* polymorphisms and haplotypes exist that act as genetic modifiers and may be associated with an increased risk for the development of neural crest cell derived disorders. These polymorphisms may

interact with other genetic variants or with MEN2-associated germline mutations modulating the disease phenotype or age of onset. Since polymorphisms are comparatively common in the population, they may present a much higher attributable risk in the general population than rare mutations in high penetrance cancer susceptibility genes such as *RET*. Here we review the present findings for the different endocrine syndromes/tumors.

MEN2 and FMTC

It has been suggested that the *RET* polymorphisms G691S and S904S have a modifier effect on the age of onset of MEN2A (44) and the same has been concluded for the SNP L769L in combination with the FMTC germline mutation Y791F (45).

Sporadic medullary thyroid cancer

Several *RET* polymorphisms have been described in association with sporadic MTC. The SNPs S836S (46, 47) and IVS1-126G→T (9) were both found overrepresented and apparently associated with the somatic mutation M918T. Other studies however did not confirm this finding (45, 48-50). A specific haplotype (the so-called CGGATGCCAA haplotype), harboring among others the SNPs G691S and S904S (50), appears associated with sporadic MTC. Both SNPs have previously been associated with MEN2A (44, 51). G691S has been suggested to create a new phosphorylation site, affecting downstream signalling (44) or to change the secondary structure of *RET* (50). SNP IVS14-24G→A, originally interpreted as an HSCR causing mutation (52), has also found been more frequently in sporadic MTC patients and in subjects with moderately elevated serum calcitonin concentrations after calcitonin stimulation tests, than in a control group (45). In another study however, IVS14-24G→A appeared not to be associated with either HSCR or sporadic MTC (53). Identification of a haplotype with a protective effect against sporadic MTC has recently be claimed (50).

Sporadic pheochromocytoma

A low-penetrance *RET* haplotype comprising the wild-type allele at IVS1-126 and IVS1-1463, with a 16-base pair intron 1 deletion 5' of these SNPs is strongly associated with and overrepresented in sporadic pheochromocytoma (54). The significant association between

genotype and the patients' age of diagnosis suggests that the additive effect of the haplotypes can modulate the age of onset of the disease.

All this shows that several different *RET* polymorphisms and haplotypes have been associated with different MEN2-related endocrine tumors. However, for almost all polymorphisms the findings are inconsistent, maybe due to the small sample sizes analyzed. Therefore, the significance and mechanism of action of these potential genetic modifiers remain to be demonstrated.

Hirschsprung's disease

Hirschsprung disease (HSCR) is a complex genetic disorder characterized by aganglionosis of a variable length of the distal gastrointestinal (GI) tract (55). Genetic dissection has led to the identification of 10 genes and 4 loci for Hirschsprung disease susceptibility (55) of which *RET* has been shown to be the most important one. Coding sequence mutations (CDS) in *RET* are responsible for a dominant form of HSCR (with incomplete penetrance) and mutations are found in up to 50% of the familial cases and 15% of the sporadic cases (56). Besides CDS there is ample evidence that also non-coding RET variants play a major role in HSCR development. In association studies performed in populations from in The Netherlands, Italy, France, Spain, USA and Hong-Kong, the same disease-associated haplotype was observed in the 5'region of the RET locus in 56%-62% patients of European descent (including Americans) (57, 58, 59, 60, 61, 62). In Asian patients, the same haplotype was also present, though the frequency was much higher (85%) (63, 64). The common haplotype spans a 27 Kb region (4 Kb region of 5' UTR, exon 1, 23 Kb intron 1 and exon 2 of *RET*) which strongly indicates the occurrence of regulatory mutations either in the promoter region or intron 1 of *RET*. Additionally, most of the patients that were found to carry the HSCR-haplotype were homozygous for it, suggesting dose-dependent action of the unknown mutation(s). Several groups have characterized candidate disease-associated polymorphisms (58, 63, 64, 65, 66). Two promoter SNPs - rs10900296 and rs10900297 (SNP-5 G>A and SNP-1 A>C, respectively), located just upstream of the *RET* transcriptional start site were shown to reduce promoter activity in luciferase reporter assays (58, 63), though others found this effect to be cell line dependent (66). Based on association studies, functional assays and comparative genomics two other groups reported two SNPs (rs2435357 and rs2506004) located closely to each other in intron 1 as probable disease causing variants (64, 65). In

particular the latter approach seems to be useful in judging the functional relevance of non-coding mutations, as disease-associated variants are likely located in regulatory transcription factor binding sites, which are in regions conserved among the different vertebrate species. The two SNPs discussed here proved to lie in a single region highly conserved among different mammalian species but also in other vertebrates such as avians. They cause disruption of a putative enhancer-binding site (rs2506004-*ETV4* enhancer site) or could interrupt proper binding of other factors to sites located close by (rs2435357-RARE elements). Mutant alleles of these two SNPs show the highest values for association in patients and the region containing the SNPs shows reduced promoter activity in luciferase assays (64). Grice et al. (67) constructed a *LacZ* reporter mouse with the MCS+9.7 *RET* intron 1 region was constructed, which carried rs2435357 and rs2506004 SNPs in the context of the *hsp68* promoter. Interestingly, MCS+9.7 driven *LacZ* expression resembled the temporal and spatial expression of *RET* during embryonic development, which is a good indication of the relevance of this particular region in the regulation of *RET* expression. Nevertheless, Grice et al. (67) could not identify transcription factor(s) binding to this sequence. In addition to already mentioned RARE elements located on each side of the rs2435357 SNP, a binding site for the SRF protein was predicted to overlap with SNP rs2435357 locus (67). The role of these potential transcription factor binding sites needs further investigation.

The presented data indicate the existence of common, regulatory mutations present in *RET*. Figure 5 presents a classification of HSCR associated *RET* mutations and their dysfunctional mechanisms of action. A large number of HSCR patients do not carry any (RET) mutation. In these patients one may suspect presence of mutations at other loci, either in coding or in regulatory sequences causing the disease.

RET as a central player in HSCR development

HSCR-associated mutations have so far been found in genes belonging to *RET* and *EDNRB* receptors signalling pathways (68). At first, these two signalling pathways seemed to be completely unrelated. There is, however, growing evidence of the existence of direct interaction between these two key signal transduction routes in enteric nervous system ENS) development, both on the genetic and the biochemical level (61, 69, 70).

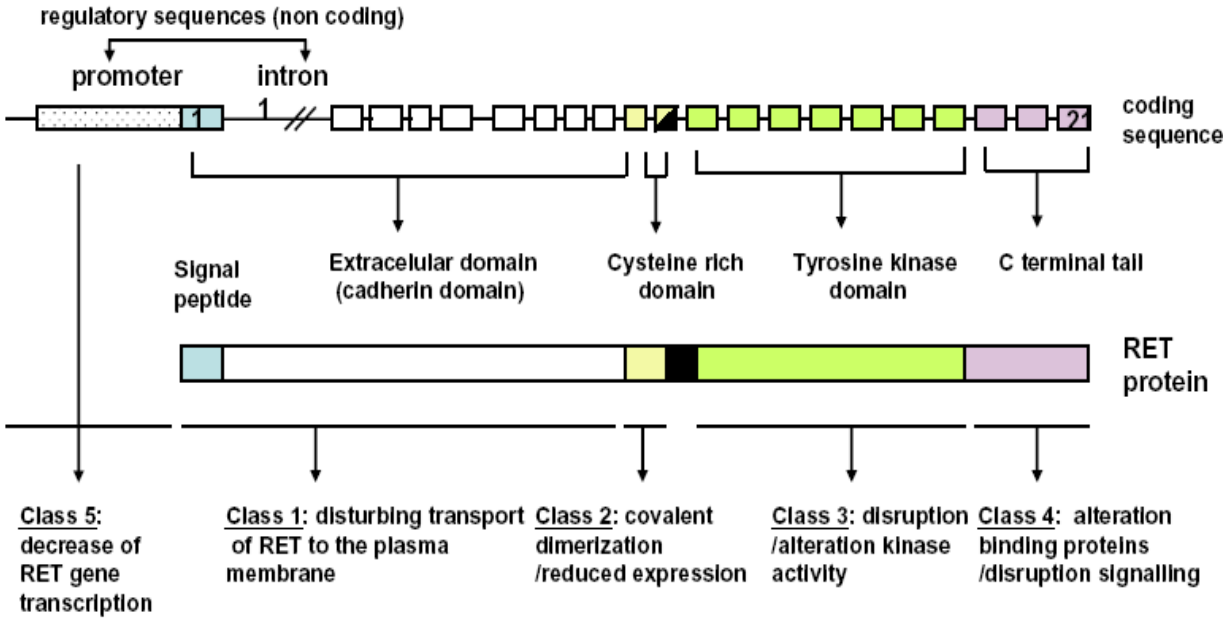

Figure 5. Classification of HSCR associated RET mutations.

Schematic representation of the different types of *RET* mutations associated with HSCR, the domains of the gene/protein affected and their functional consequences are depicted: Class 1: mutations affecting coding sequences in the extracellular domain of RET resulting in disturbed transport of RET to the plasma membrane during translation of the protein. Class 2: mutations affecting the cystein-rich domain of RET resulting in covalent dimerization of the RET protein and reduced localization of RET at the plasma membrane. Class 3: mutations targeting the kinase domain of RET causing the disruption or alteration of the catalytic activity of the receptor. Class 4: mutations located in the C-terminal tail causing alteration of binding proteins and hence disruption of signaling. E) class 5: mutations located within regulatory sequences (promoter and intron 1) which cause reduced *RET* transcription.

In the population of Old Order Mennonites specific alleles of *RET* and *EDNRB* loci appeared non-randomly associated with HSCR and much more often jointly transmitted to the patients than expected (61). In these Mennonites, who form an isolated population with an HSCR incidence of 1/500, an *EDNRB* mutation proved the major risk factor (71). Therefore, HSCR patients in this population differ from those in other populations. However, the "Mennonite" *RET* locus haplotype itself is not different from the common HSCR haplotype of patients in general. The linkage disequilibrium block present at the *RET* locus likely acts as a modifier of the mutated *EDNRB* locus and increases the penetrance of this mutation (61). To test the functional connection between *RET* and *EDNRB* pathways, intercrossed mice have been generated from already existing strains (Ret^{-}/Ret^{+}, $Ednrb^{s}/Ednrb^{s}$ and $Ednrb^{s\text{-}l}/Ednrb^{s}$)

resulting in compound heterozygous offspring for *RET* null allele (truncating deletion at Lys-748) and loss of function *EDNRB* (*s* – piebald, s^l – piebald lethal) alleles (69). In particular, the combination of mice *Ret* and *Ednrb* genotypes (Ret^-/Ret^+; $Ednrb^s/Ednrb^s$) better explained HSCR transmission and phenotype as observed in humans than did the independent mutants. This would indicate that interactions exist between *RET* and *EDNRB* pathways. Furthermore, it was shown that 100% of the male offspring with this genotype was affected and that although similarly all females manifested variable length of the aganglionic colon, 30% had reduced clinical expression of HSCR, thus showing a sex bias resembling the one observed in humans. The spatial and temporal regulation of neural crest migration and ENS formation by RET and EDNRB transduction routes has been investigated by Barlow et al. (70). Heterozygous mice strains expressing Ret^{51} and $Et\text{-}3^{ls}$ alleles and intercrosses were examined. Mice only expressing the Ret^{51} allele manifest aganglionosis of the distal gut, resembling the HSCR phenotype and provide a good model to study RET signalling in ENS development in a dose-dependent fashion (4). $Et\text{-}3^{ls}$ is a null allele of an EDNRB ligand and causes in homozygous form distal intestinal aganglionosis (72). Double homozygotes for Ret^{51} and $Et\text{-}3^{ls}$ caused total intestinal aganglionosis in 70% of the cases, indicating strong interaction between these two loci. cAMP-dependent Protein Kinase A (PKA) was pinpointed as a likely candidate that mediates interactions between the RET and EDNRB pathways. ET-3 is thought to act by reducing the activity of PKA, as a specific PKA inhibitor mimics the effect of ET3 (proliferation and inhibition of migration) on neural crest cells (70). One of the possible targets of PKA action on the RET pathway is RET serine 696. Phosphorylation of this residue by PKA promotes formation of lamellipodia in neuroectodermal cells, stimulating migration (25).

Taken together, the genetic and biochemical evidence, the central role of RET in ENS development and Hirschsprung disease is unquestionable. Nevertheless, regulation of RET signalling by other receptors, growth and transcription factors along the developing gut may be necessary for proper ENS development.

HSCR combined with MEN2

Mutations in extracellular cysteine residues 609, 611, 618 and 620 that primarily were considered as specific for MEN2A and FMTC have also been identified in (sporadic) HSCR patients and in MEN2A families in which MEN2A or FMTC sometimes segregated with

HSCR (73). These mutations have a dual impact on RET. On one hand they constitutively activate RET through the formation of covalent dimmers, but on the other hand they result in a drastic reduction of RET expression at the plasma membrane. This leads to an uncontrolled proliferation of the thyroid C-cells, as seen in MEN2A and FMTC, and in apoptosis of enteric neurons, as seen in HSCR. The reason that C-cells hyperproliferate and enteric neurons undergo apoptosis is likely due to differences in sensitivity and required thresholds for RET-mediated signalling (73), as it was recently shown for the C634R and C620R *RET* mutations **(**74).

ONCOGENIC RET ACTIVATION AND SIGNALLING IN MEN2.

Despite the clear genotype-phenotype correlation of mutations found in MEN2, the molecular mechanisms connecting the mutated receptor with the different disease phenotypes are far from completely understood.

Specific germline missense mutations giving rise to an activated RET protein cause MEN2 and FMTC. These mutations can affect the extracellular cysteine-rich domain or the intracellular tyrosine kinase domain of RET (Fig. 4, ref 16). Mutations in the extracellular cysteine-rich domain result in the replacement of a cysteine residue by another amino acid, subsequently giving rise to the loss of an intramolecular disulfide bond. As a consequence one cysteine residue becomes available for the formation of an intermolecular disulfide bond between two (mutated) RET monomers, resulting in a constitutively activated receptor (12). Intracellular mutations are generally associated with FMTC and MEN2B. The way these mutations affect activation of the receptor is totally different compared to the mutations in the cysteine rich domain. They signal as monomeric oncoproteins independent of GDNF (75). These intracellular mutants not only show an altered catalytic activity but also and altered substrate specificity, as they phosphorylate substrates preferred by cytoplasmic tyrosine kinases, such us Src and Abl (12).

In figure 6, a schematic representation of the different mechanism of receptor activation by wild type and MEN2-associated RET mutants is presented.

In addition to the different oncogenic mechanisms of RET activation observed in MEN2, a different pattern of receptor autophosphorylation has been shown for RET-MEN2A and RET-MEN2B oncoproteins (76, 77).

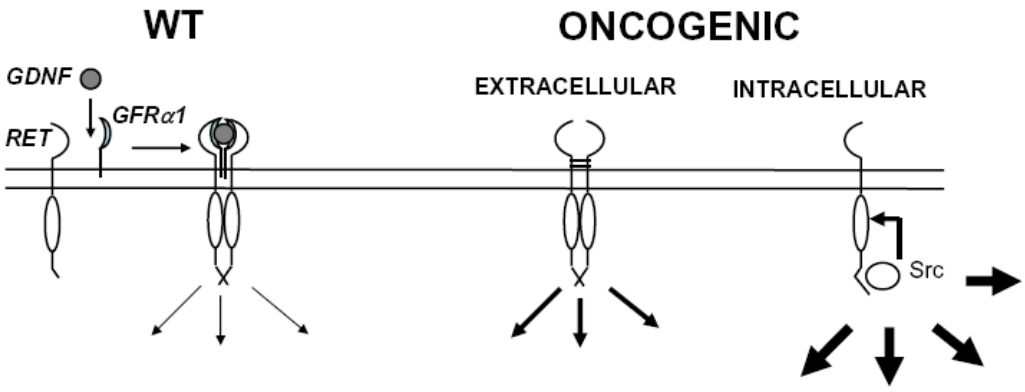

Figure 6

Different mechanism of receptor activation by wild type RET and MEN 2-associated RET mutations.

A) ligand regulated activation of wild type RET: the ligand (GDNF) first binds to the GPI-anchor co-receptor (GFRα-1), followed by RET recruitment to form a macromolecular complex receptor.

B) Constitutive activation of RET by mutations affecting the cysteine rich domain cause the covalent dimerization and result in ligand-independent constitutive signaling of the receptor.

C) Mutations affecting the tyrosine kinase domain of RET result in monomeric oncoproteins with altered catalytic activity and altered substrate specificity, as they seem to preferentially recognize substrates of cytoplasmic tyrosine kinases such us Src and Abl.

As a consequence, different sets of phosphotyrosine mediated/activated downstream signalling pathways could be triggered by RET mutants specific for a disease phenotype explaining the observed distinct clinical features.

Studying the mechanisms by which specific RET mutants differentially activate downstream signalling pathways, their biological implications, and the structural basis associated with the different MEN2- RET oncoproteins, will help us to understand the molecular basis of this disease and to find new therapeutic strategies (see Discussion and future perspectives).

CONCLUSION

In conclusion, the *RET* gene is an excellent example of how mutations, alone or as part of a polygenic model give rise to different (inherited) human diseases by altering the signalling properties and transcriptional regulation of the protein it encodes. The unraveling of the genetic and molecular mechanisms underlying different RET-related neural crest disorders has not only been a success in the history of genetic medicine, but by helping us understand how these different diseases develop may also contribute to the development of new therapeutic strategies.

Table 1
Subdivision of RET mutations according to the position in the coding sequence, the consequences of the mutations and the predisposing phenotype (33-35).

		Codons mutated /position of the mutation	*Consequences of the mutation*
Activating MEN2 mutations	Level 1	Mutation at codons 609, 768, 790, 791, 804, 891	Mild activating RET mutations predisposing to FMTC
	Level 2	Mutation at codons 611, 618, 620, 634	Moderate activating RET mutations predisposing to FMTC or MEN2A
	Level 3	Mutation at codons 883, 918,	Aggressive RET mutations predisposing to MEN2B
Inactivating HSCR mutations	Class 1	In the extracellular domain	Disrupt RET maturation/transport to the membrane
	Class 2	Mutation at codons 609, 611, 618, 620	Covalent dimerization. Co-segregation of HSCR and MEN 2/FMTC
	Class 3	In the catalytic domain	Abolishing catalytic activity
	Class 4	Mutations surrounding residue Y1062	Interfere with substrates binding to this tyrosine
	Class 5	In regulatory sequences	Changing the expression of *RET*

Table2
Biological properties of different RET mutations in NIH-3T3 cells and mice.

Codon mutated in RET	*Transforming activity in NIH-3T3*	*Maturation of the protein*	*Promoter/knock-in/knock-out*	*Mouse phenotype*	*Ref.*
C609Y	+	++			36
C611Y	+	++			36
C618Y	+	++			36
C620R	++	++			36
C630R	++				36
C634R	+++/+++++	++++	Calcitonine gene related peptide/calcitonine promoter	MTC	37
C634R			Moloney murine leukaemia virus repeat	C-cell hyperplasia/MTC 50% Mammary or parotic gland adenocarcinoma	38
A883F	+++++				39
M918T	+++++		Dopamine-hydroxilase promoter	Bening neuroglial tumors C-cells and chromaffin cells hyperplasia	39/40
			Knock-n (heterozygous)	C-cells and chromaffin cell hyperplasia (early on-set) and male reproductive defects	41
			Transgenic mice/calcitonine promoter	MTC(3/8)	42
RET			Targeted disruption RET (knock-out)	Renal dysgenesis and lack of enteric neurons	43

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Chapter 2

RET-FMTC mutants Y791F and S891A activate a Src/JAK/STAT3 pathway, independent of GDNF

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ABSTRACT

The *RET* proto-oncogene encodes a receptor tyrosine kinase whose dysfunction plays a crucial role in the development of several neural crest disorders. Distinct activating *RET* mutations cause Multiple Endocrine Neoplasia type 2A (MEN2A), type 2B (MEN2B) and Familial Medullary Thyroid Carcinoma (FMTC). Despite clear correlations between the mutations found in these cancer syndromes and their phenotypes, the molecular mechanisms connecting the mutated receptor to the different disease phenotypes are far from completely understood. Luciferase reporter assays in combination with immunoprecipitation, western and immunohistochemistry analyses were performed in order to characterize the signalling properties of two FMTC associated *RET* mutations, Y791F and S891A, respectively, both affecting the tyrosine kinase domain of the receptor.

We show that these RET-FMTC mutants are monomeric receptors which are autophosphorylated and activated independently of GDNF. Moreover, we demonstrate that the dysfunctional signalling properties of these mutants, when compared to wild type RET, involve constitutive activation of STAT3. Furthermore, we demonstrate that STAT3 activation is mediated by a signalling pathway involving SRC, JAK1 and JAK2, differing from STAT3 activation promoted by RETC634R which previously was found to be independent of SRC and JAKs. Three-dimensional modeling of the RET catalytic domain suggested that the structural changes promoted by the respective amino acids substitutions lead to a more accessible substrate and ATP binding monomeric conformation. Finally, immunohistochemical analysis of FMTC tumor samples support the *in vitro* data, as nuclear localized, Y705-phosphorylated STAT3 as well as a high degree of RET expression at the plasma membrane was observed.

INTRODUCTION

The *RET* proto-oncogene encodes a receptor tyrosine kinase which is expressed in neural crest derived cells, including enteric and sympathetic neurons, adrenal chromaffin cells and parafollicular c-cells of the thyroid gland (1). Ligands of the RET receptor are members of the Glial cell Derived Neurotrophic Factors (GDNF) family: GDNF, Neurturin, Persephin and Artemin. They are able to activate RET in the presence of GFR-α $_{(1-4)}$ glycosyl phosphatidyl-inositol (GPI) anchored co-receptors (2-7).

Activation of RET by its ligand results in trans-phosphorylation of multiple tyrosine residues that, in turn act as docking sites and interact with specific adaptor proteins to trigger downstream signalling pathways crucial for the survival and differentiation of neural crest derived lineages, as well as kidney organogenesis (8-10). Phosphotyrosine residues 905, 981, 1015, 1062 and 1096 serve as docking sites for Grb7/10, SRC, Phospholipase Cγ (PLC-γ), SHC/ENIGMA/FRS2/IRS1-2/DOK4-5 and GRB2, respectively (11-15). In general, the signalling pathways activated by RET include RAS-MAPKs, PI3K, c-Jun N-terminal Kinase (JNK), p38, ERK-5, PLC-γ and STAT3 (16-18). Additionally, RET can activate members of the Rho family of GTPases, including RHO, RAC and CDC42, that are involved in the reorganization of the cytoskeleton and are responsible for cell motility and morphology (19-20). Tyrosine residues located at the catalytic domains such as Y687, Y806, Y809, Y900, Y826 and Y1029 have also been shown to be phosphorylated in response to RET activation but their downstream signalling pathways have not been elucidated yet (21-22).

Specific germline missense mutations in the *RET* proto-oncogene leading to a constitutive activation of the receptor cause a dominant inherited cancer syndrome called Multiple Endocrine Neoplasia type 2 (MEN2) and a familial form of Medullary Thyroid Cancer (FMTC). Depending on the affected tissues, two different clinical subtypes of MEN2 can be recognized. MEN2A is characterized by Medullary Thyroid Carcinoma (MTC), pheochromocytoma and hyperplasia of the parathyroid. MEN2B is characterized by MTC, pheochromocytoma, but instead of hyperplasia of the parathyroid, patients develop neuromas in tongue, lips and eyelids, and also intestinal ganglioneuromas. In FMTC, only the c-cells of the thyroid become malignant (23).

Mutations located in the cystein rich domain of RET (codons 609, 611, 618, 620, 630, 634) give rise to MEN2A and FMTC. Distinct mutations found in the tyrosine kinase domain of the receptor can give rise to FMTC (codons 768, 790, 791, 804, 891) or to MEN2B (codons 883, 918) (24). Interestingly, mutations in the cystein rich domain (codons 609, 611, 618, 620) are not only found in families with MEN 2A/FMTC but also in patients with Hirschsprung's disease (HSCR), a congenital malformation characterized by an absence of enteric ganglia cells in the distal part of the colon, or patients having a combination of MEN2 and HSCR (25-26)

As mentioned, FMTC mutations can be localized both in the cysteine rich motif as well as in the catalytic domain of the protein. In this report, we investigate the activation and downstream signalling routes triggered by two RET-FMTC mutants, Y791F (27) and S891A (28), both mutations are localized in the tyrosine kinase domain of the receptor.

MATERIALS AND METHODS

Cell lines and cell culture reagents

SK-N-SH, COS-7, HepG2, HEK293 and MZ-CRC-1 (29) cells were grown in Dulbecco´s Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Calf Serum (FCS) (Gibco), 100 μg/ml penicillin, 100 μgr/ml streptomycin and 2 mM L-glutamine. MTC-TT cells (30) were grown in RPMI supplemented with 15% FCS, (Gibco), 100 μg/ml penicillin, 100 μgr/ml streptomycin and 2 mM L-glutamine.

Cells were stimulated with 50 or 100 ng/ml GDNF (Glia cell line Derived Neurotrophic Factor) where indicated (Prepotech, Rocky Hill, NJ, USA). Different inhibitors were employed: U0126 (Promega, Madison, USA), AG1296, AG490 and PP2 were purchased from Calbiochem (San Diego, USA).

Expression and reporter plasmids

The pCR-CMV-RETwt (RETWT) plasmid encoding the short form of the human *RET* proto-oncogene cDNA was used to create the RETS891A and RETY791F mutants by site directed mutagenesis according to the manufacturer's instructions (Stratagene, La Jolla, USA) using the following forward and reverse primers:

RETS891A 5'-GGAAGATGAAGATTGCGGATTTCGGCTTGTCCC-3'

RETS891A 3'-GGGACAAGCCGAAATCCGCAATCTTCATCTTCC-5',

RETY791F 5'-CCACATGTCATCAAATTGTTTGGGGCCTGCAGCCAGC ATGGCCC-3

RETY791F 3'-GGGCCATGCTGGCTGCAGGCCCCAAACAATTTGATG ACATGTGG-5.

Following mutagenesis, all constructs were fully sequenced. The Mercury™ Pathway Profiling Luciferase System #K2049-1 (Clontech, Palo Alto, USA) containing several pathway-specific reporter constructs was used. We also tested the pIRE-ti-Luc (containing two copies of the IL-6 response element of the ICAM-1 promoter) and pIREmut-ti-Luc reporters (17). The dominant negative (DN) JAK1 and JAK2 constructs were kindly provided by Dr. Lu- Hai Wang and were described previously (31). The DN-SRC plasmid was a gift of Dr. Yung H. Wong, and was described previously (32).

The pSG5-STAT3α and pGS5-STAT3β expression plasmids were a gift from Dr. R. de Groot, Dept. of Pulmonary Diseases, Utrecht.

Luciferase reporter assays

Cells were transfected using the calcium phosphate precipitation method as described previously (17).

Western blotting and immunoprecipitation

The following antibodies (1:1000) were used in protein analyses: Phosphotyrosine 4G10 (Upstate, New York, USA), RET (H-300), STAT3 (C-20) (Santa Cruz) and Phospho STAT3 (Tyr705) (Cell Signaling Technology, New England Biolabs, UK). Western blotting and immunoprecipitation were performed as described previously (17).

Blue native SDS-PAGE

Cells were lysed on ice with lysis buffer (10mM Tris-Cl pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P40, 1mM sodium vanadate, 10 ugr/ml aprotinin, 2 ug/ml leupeptin, 0.2 mM PMSF, 10 % glycerol) and blue native-PAGE was performed as described previously (34).

Immunohistochemistry

From the tumor cell-lines MTC-TT and MZ-CRC-1, about $2x10^6$ cells were fixed with buffered formalin pH 7.4 for 1 hour at 4ºC. After washing with phosphate buffered saline (PBS), 150 µl plasma (Sigma coagulation control level 1; C7916) was added, followed by 50 µl thromboplastine/Ca^{2+} (Sigma; T7280). This was incubated for 10 minutes at 37ºC in a water bath. The gel was embedded in paraffin.

Immunohistochemistry was performed on a total number of 5 tumor samples of independent patients all carrying the Y791F-RET mutation, and it was performed as described previously (35).

Structural modeling of RET cytoplasmic tyrosine kinase domain

The crystal structure of the human fibroblast growth factor receptor 1 FGFR1K (PDB accession code 1FGK) determined at 2.0 Å resolution (36) was selected as template to construct a model of the RET tyrosine kinase domain. The tyrosine kinase domains of FGFR1K and RET (RETK) share 50% amino acid sequence identity. The homology models of the wild-type and mutant RETKs were constructed using MODELLER (37)

version 7 as implemented in DS Modeling 1.1 (Accelrys Inc, San Diego, CA) using standard settings. The quality of the models was evaluated using Whatif (38) and Procheck (39). Quality parameters of the models, such as structure Z- scores and G-factors, were comparable to those of the template structure. A short energy minimization using constraints on the backbone atoms was performed using the CHARM (40) module of DS Modeling 1.1.

RESULTS

GDNF-dependency of RETWT, RETY791F and RETS891A signalling

In order to determine the signalling properties of the wild type RET receptor in response to GDNF, SK-N-SH cells, which are GFRα-1 positive (data not shown), were transfected with an RETWT expression plasmid in combination with different luciferase reporter constructs. GDNF treatment (100 ngr/ml) resulted in activation of the SRE, NFkB and, to a lesser extent, the IRE reporters (Fig. 1A), where the HSE, AP-1 and GRE reporters were not activated. When SK-N-SH cells were transiently transfected with either RETY791F or RETS891A expression plasmids in combination with the different reporters, activation of the SRE, NFkβ and IRE reporters was observed. The level of IRE reporter activation was however clearly higher than that observed with RETWT (Fig. 1B and C). GDNF did not further enhance reporter activation by RETY791F and RETS891A, indicating that these mutant RET receptors signalling is independent of GDNF. To validate the responsiveness of the reporter constructs used, the HSE, AP1 and IRE reporters were treated with a heat shock (43˚, 30 min.), UV (45 min.) or co-transfected with v-SRC, respectively. An increase in reporter activity in response to these treatments was observed (HSE: 20-fold, AP-1: 3-fold and IRE: 12-fold; data not shown).

To determine whether the observed GDNF-independent signalling of these FMTC mutants is reflected by constitutive receptor autophosphorylation, COS-7 cells were transfected with RETWT, RETY791F and RETS891, respectively. Cell lysates were subjected to anti-RET immunoprecipitation (IP) followed by western analysis (IB) using anti-phosphotyrosine and anti-RET antibodies. Whereas tyrosine phosphorylation was not observed in the wild type RET receptor; strong GDNF-independent phosphorylation was observed in both FMTC mutants. Tyrosine phosphorylation of both mutant receptors was not further increased by GDNF stimulation (data not shown).

These results supported the findings obtained in the reporter assays where these mutants activated reporter activity in the absence of GDNF.

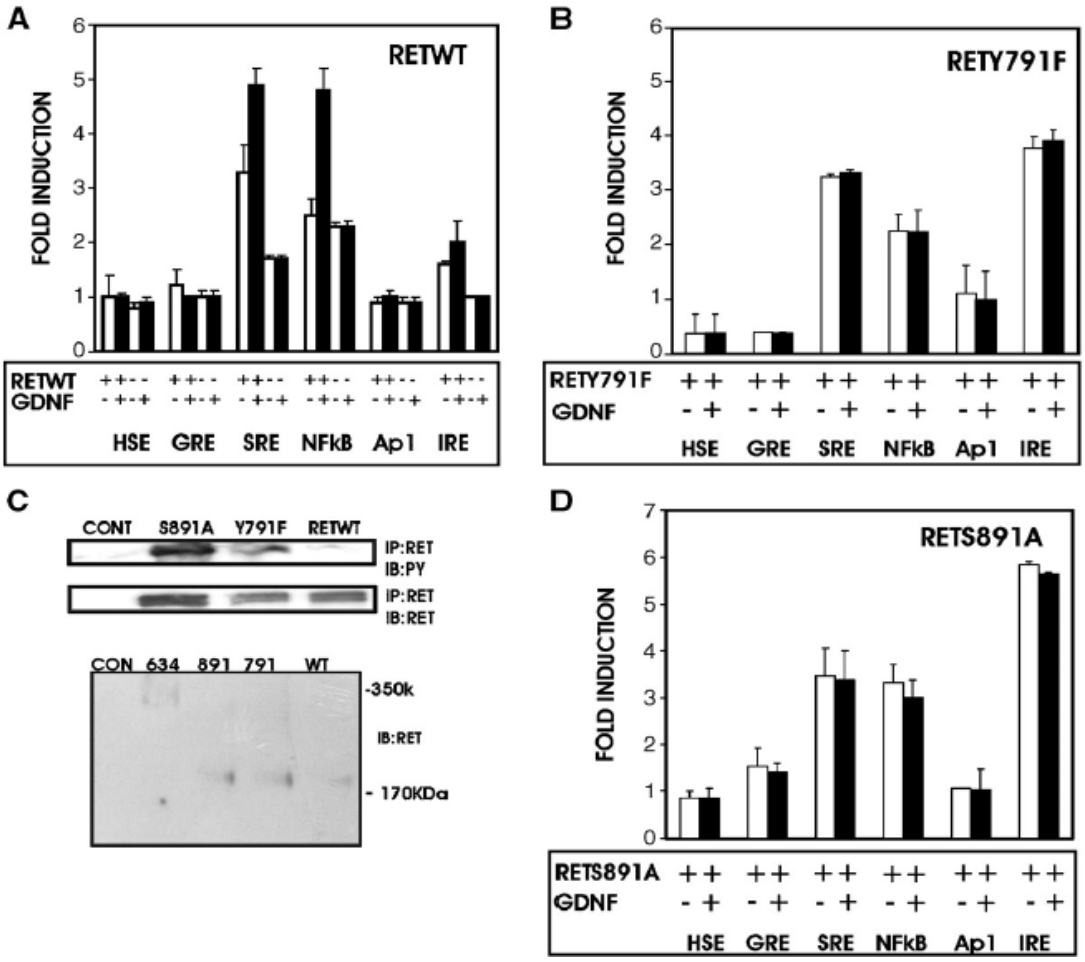

Figure 1

Signalling properties of RETWT, RETY791F and RETS891A

A, B & D) SK-N-SH cells were transfected with RETWT, RETY791F or RETS891A in combination with different reporter plasmids and stimulated with GDNF as indicated. Normalized reporter activity is given as mean fold induction +/- S.D. of triplicates.

C) COS-7 cells were transfected with RETWT, RETY791F and RETS891A as indicated. Cell lysates were subjected to RET immunoprecipitation (IP) and immunoblotting (IB) with an anti-phosphotyrosine and RET antibodies, respectively. Moreover, the same cell lysates were analyzed using blue native PAGE followed by western analysis using an anti-RET antibody.

Monomeric and dimeric RET proteins

Iwashita and co-workers demonstrated that RETS891A functions as a monomeric oncoprotein (41). Whether the Y791F mutant signals as a monomer or as a dimmer is at present unknown (24). In order to determine the mono/di-meric state of RETY791F, COS-7 cells were transfected with wild type and mutant RET expression plasmids, after which cell lysates were analyzed using blue native gel electrophoresis and western blotting. RETC634R, a known dimmer, RETS891A (monomeric) and

RETWT (monomeric) were included as controls. From these studies we concluded that, like the S891A mutant, the Y791A mutant functions as a monomeric receptor (Fig. 1E).

STAT3 activation by RETY791F and RETS891A

GDNF-independent activation of the IRE reporter by RETY791F and RETS891A was observed. As the IRE reporter contains specific binding sites for members of the STAT family of transcription factors (42) and since it has previously been shown that the RET receptor contains two STAT3 specific docking sites (Y752 and Y928) (17), we determined whether the observed IRE transactivation by RETY791F and RETS891A was mediated by STAT3. For this purpose, SK-N-SH cells were transiently transfected with an IRE-Luc reporter in combination with STAT3α and RETS891A. In case the IRE reporter was co-transfected with RETS891A, a 5-fold increase in IRE-reporter activity was observed. When STAT3α was also included, IRE-reporter activation increased to 20-fold. Overexpression of STAT3α alone had no effect on reporter activity (Fig. 2A). In case STAT3β, a splice variant of STAT3α, which lacks the C-terminal tail of the molecule, was co-transfected, RETS891A induced activation of the IRE luciferase reporter progressively decreased when increasing amounts of STAT3β were used (Fig. 2A). These results indicate that activation of the IRE reporter by RETS891A is mediated by STAT3. Furthermore, when an IRE reporter was used in which the STAT3 binding sites are mutated (IRE-mut-Luc), no reporter activation was observed upon co-transfection with RETS891A. To confirm that these results are not cell type dependent, we performed similar experiments using HepG2 and COS-7 cells. In both cell lines, again potentiation of IRE activity in response to RETS891A by STAT3α and inhibition of IRE-Luc activation by STAT3β was observed (Fig. 2B). When RETY791F was used, similar results as observed with RETS891A were obtained (Fig. 2D).

To further substantiate that FMTC-RET mutants can activate STAT3, the effect of these mutants on STAT3 Y-705 phosphorylation was determined. HEK293 cells were transiently transfected with RETWT, RETY791F and RETS891A expression plasmids, treated in presence (50 ngr/ml) and in the absence of GDNF, and cell lysates were western analyzed.

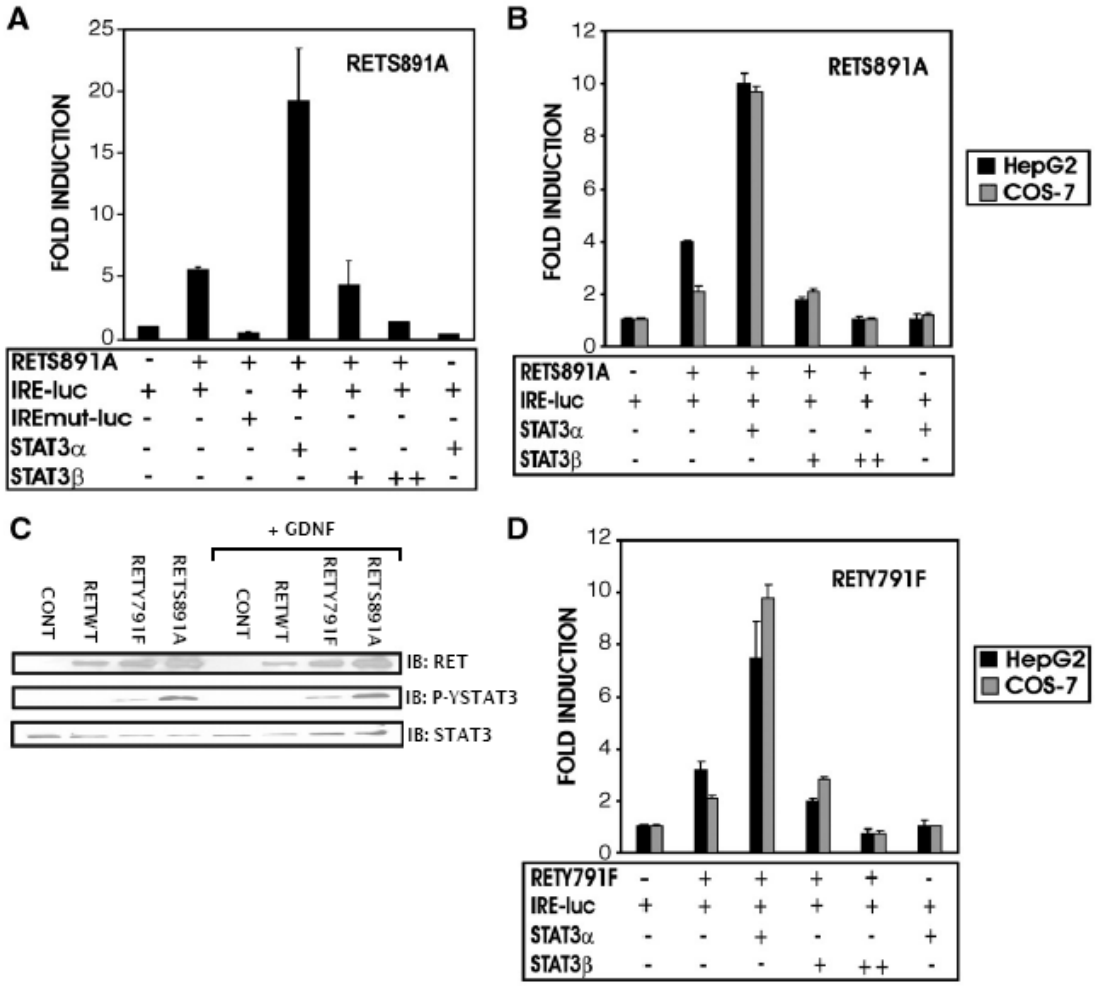

Figure2

RETY791F and RETS891A induce STAT3 transactivation

A,B & D) SK-N-SH (panel A), HepG2 and COS-7 cells (panels B and D) were transfected with IRE and IRE-mut reporter constructs in combination with STAT3 α, STAT3β, RETY791F and RETS891A expression plasmids as indicated. C) HEK293 cells, transfected with RETWT, RETY791F and RETS891A, and treated or non-treated with GDNF, respectively. Cell lysates were western analyzed using antibodies against phosphoY705-STAT3, STAT3 and RET, respectively.

Both FMTC mutations (RETY791F and RETS891A) induced an increase in STAT3-Y705 phosphorylation levels where overexpression of RETWT did not result in STAT3 phosphorylation. STAT3 tyrosine phosphorylation induced by both FMTC mutants was not further increased by GDNF stimulation (Fig. 2C).

RETY791F and RETS891A activate STAT3 through a Src, JAK1 and JAK2 dependent pathway

To determine whether the activation of STAT3 by RETY791F and RETS891A was mediated by the tyrosine kinase domain of RET exclusively (17) or whether other cytoplasmic kinases contribute to this process (43-44), different inhibitors of known STAT3 activators were used in SK-N-SH cells transiently expressing RETS891A in combination with the IRE-luc reporter (Fig. 3A). First, we showed that the tyrosine kinase inhibitor AG1296 (20μM and 40 μM) reduced IRE reporter activity in a dose dependent manner suggesting that the kinase domain of RET indeed is involved in STAT3 activation. When treating cells with JAK inhibitor AG490 (15μM and 20 μM), a dose-dependent decrease in IRE reporter activity was observed, suggesting that besides RET also JAK is involved in the activation of STAT3. Moreover, the treatment of cells with SRC inhibitor PP2 (30 nM) led to a reduction of IRE reporter activity as well. Furthermore, the MEK inhibitor U0126 (10 μM) partially inhibited IRE reporter activity, suggesting the involvement of a MEK-MAPK pathway in regulating the transcriptional activity of STAT3 through phosphorylation of serine 727 (45). The same experiment was performed with STAT3α overexpression, and the same results were obtained (data not shown). None of the inhibitors however completely abolished IRE-reporter activation by FMTC-RET, suggesting that these molecules are interplaying during signaling. To further confirm the involvement of Src and JAKs, we determined the effect of dominant negative (DN) forms of SRC (32), JAK1 (containing a 3 amino acids change in the conserved region VIII of the kinase domain) and JAK2 (containing a Lysine to Alanine mutation in the ATP binding site) (31) on IRE reporter activation by RETY791F and RETS891A. Overexpression of DN-SRC resulted in decreased IRE reporter activity by both FMTC mutations in a dose dependent manner (Fig. 3B). IRE reporter activity was also markedly decreased by overexpression of either DN-JAK1 or DN-JAK2 (Fig. 3D). These results suggest that RETY791F and RETS891A activate STAT3 through a pathway involving SRC, JAK1 and JAK2.

Similar experiments were performed with RETC634R. In contrast to the results obtained for the FMTC mutant, the SRC inhibitor PP2 does not have any effect on IRE reporter activation by RETC634R (Fig. 3C). The same results were obtained using a dominant negative SRC construct (Fig. 3C). The JAK inhibitor AG490 shows

a slight inhibition of the IRE reporter activation by C634R (Fig. 3C) but to a lesser extent than FMTC-mediated activation, which was almost completely inhibited. Using DN-JAK1 and DN-JAK2 (Fig. 3C) we were able to observe that this slight inhibition could be JAK1 dependent as no effect on the IRE reporter was observed for DNJAK2 whereas DNJAK1 was slightly inhibited. From these experiments we concluded that indeed as reported RETC634R activates STAT3 through a pathway independent of SRC and JAK2.

Immunohistochemical analysis of RET in FMTC tumor samples and tumor cell lines

Analysis of tumor sections from 5 patients carrying germline *RET* Y791F mutation with an anti-RET antibody displayed a high degree of RET staining at the plasma membrane (Fig.4 A-C). Paraffin embedded tumor cell lines MTC-TT (MEN2A) and MZ-CRC-1 (MEN2B) were also analyzed for RET expression as positive controls, showing a high degree of the RET receptor at the plasma membrane as well (Fig. 4D). MZ-CRC-1 cells as well as all tumor tissues of FMTC patients carrying the *RET* Y791F mutation, show besides the mentioned positive RET staining at the plasma membrane also cytoplasmic RET staining. In case of the MTC-TT (MEN2A) cell line, RET staining was mainly observed at the plasma membrane.

Immunohistochemical analysis of phospho Y705-STAT3 and STAT3 in FMTC tumor samples and tumor cell lines

Staining of the same tumor samples and cell lines, as mentioned in the previous paragraph, with anti-STAT3 and anti-phospho Y705-STAT3 showed in all samples high levels of phosphorylated STAT3 in the nucleus (Fig. 5A-C) whereas STAT3 was detected both in the cytoplasm and the nucleus (Fig. 5D). These results indicate strong activation of STAT3 in MTCs.

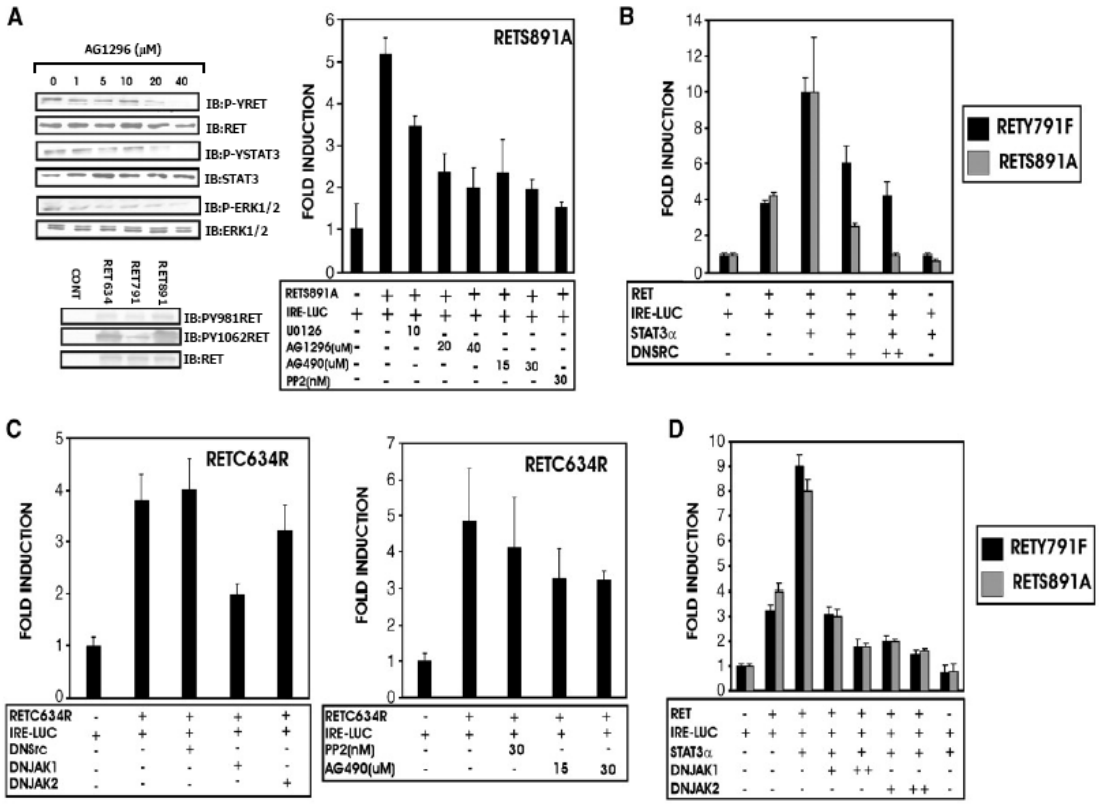

Figure 3

STAT3 transactivation by RETY791F and RETS891A requires the catalytic domain of RET, SRC and JAK1, 2

A) SK-N-SH cells were transiently transfected with the IRE-Luc reporter together with RETS891A. Twenty-four hours before harvesting, cells were treated with 10 μM U0126 (MEK inhibitor), 20μM and 40 μM AG1296 (tyrosine kinase receptor inhibitor), 15μM and 30 μM AG490 (JAK2 inhibitor) and 30 nM PP2 (SRC inhibitor) as indicated (right panel). HEK293 cells expressing RETC634R, RETY791F and RETS891A were analyzed by Western blotting using antibodies against phospho Y-918R ET, phospho Y-1062RET and RET, respectively (left panel).

B & D) HepG2 cells were transiently transfected with an IRE-Luc reporter and both RETY791F or RETS891A in combination with STAT3α, dominant negative SRC (DNSrc) and dominant negative JAK1 or JAK2 (DNJAK1, DNJAK2) expression plasmids (1 μgr and 2 μgrs, respectively) as indicated.

C) HEK293 cell were transiently transfected with both a RETC634R expression plasmid and an IRE reporter and cells were treated with PP2 (30nM) and AG490 (15 and 30 uM) as indicated.

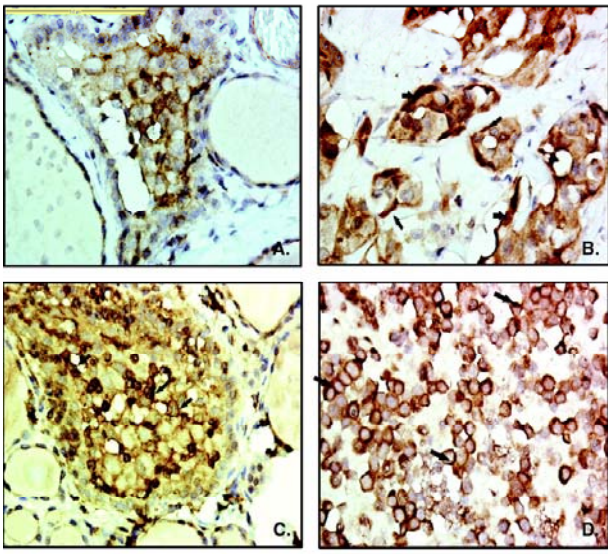

Figure 4

Immunohistochemistry detection of RET in FMTC tumors sections and tumor cell lines

A-C) 3 different medullary thyroid carcinomas (MTC) (x400), from patients carrying a germline FMTC *RET* Y791F mutation showing high degree of RET expression on the plasma membrane. D) Tumor cell line MTC-TT (X400) showing high degree of RET expression on the plasma membrane as well.

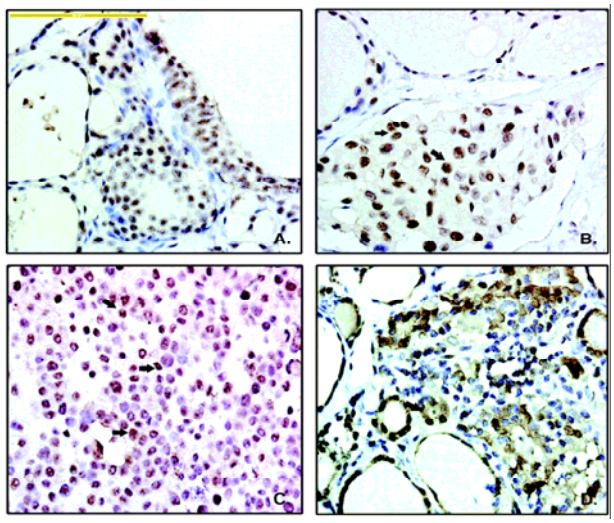

Figure 5

Immunohistochemistry detection of phospho Y705-STAT3 and STAT3 in FMTC tumors sections and tumor cell lines

A-C) Staining with a phospho Y705-STAT3 antibody (Tyr705) displayed a clear nuclear localization of the active transcription factor in medullary thyroid carcinomas (panels A-B) and in the tumor cell line MTC-TT (x400)(panel C). D) IHC performed with STAT3 antibody show a clear cytoplasmic and nuclear signal. The scale bar shows 100 μm.

Structural modeling of RET cytoplasmic tyrosine kinase domain (RETK).

Structural models of wild type and mutant RET kinase domain were obtained by comparative modeling using the tyrosine kinase domain of fibroblast growth factor receptor 1 (FGFR1) as template. The models show the characteristic bi-lobate structure of tyrosine kinases catalytic domains. Like the inactivated tyrosine kinase domains of fibroblast growth factor receptor 1 and 2, the model of wild type RETK shows a relatively open ATP binding site (Fig. 6A). Residue Ser891 is located at the beginning of the activation loop in the cleft between the N- and C-terminal lobes and just proximal to the conserved DFG motive (Residues 892-894). Comparison between the inactivated (46) and activated (47) structures of the insulin receptor kinase domain shows that at the position before the DFG motive the activation loop conformations begin to diverge. The activation loop in the inactive conformation blocks access of peptide substrate to the active site and aspartic acid 892 in RET is not properly positioned for interaction with the β-phosphate group of ATP through coordination with Mg2. Mutation of Ser891 in RET to the smaller hydrophobic alanine possibly permits the activation loop to change to a conformation compatible with peptide substrate binding, as well as a proper interaction with the ATP, in monomeric state (Fig. 6B). The other FMTC mutation, Y791F, is located in the β4 strand of the N-terminal lobe and is spatially located near the conserved WE motive and is partly solvent exposed. Phosphorylation of this residue has not been demonstrated (21). The Y791F mutation probably disrupts interactions with surrounding Glu805 and Lys789 residues, resulting in a conformational change of the loop connecting the αC helix and the β4 strand. Through the subsequent spatial rearrangement of the αC helix the conformation of the activation loop changes into the active state by interaction of αC helix residues with phenylalanine 893 of the DFG motive (Fig. 6C).

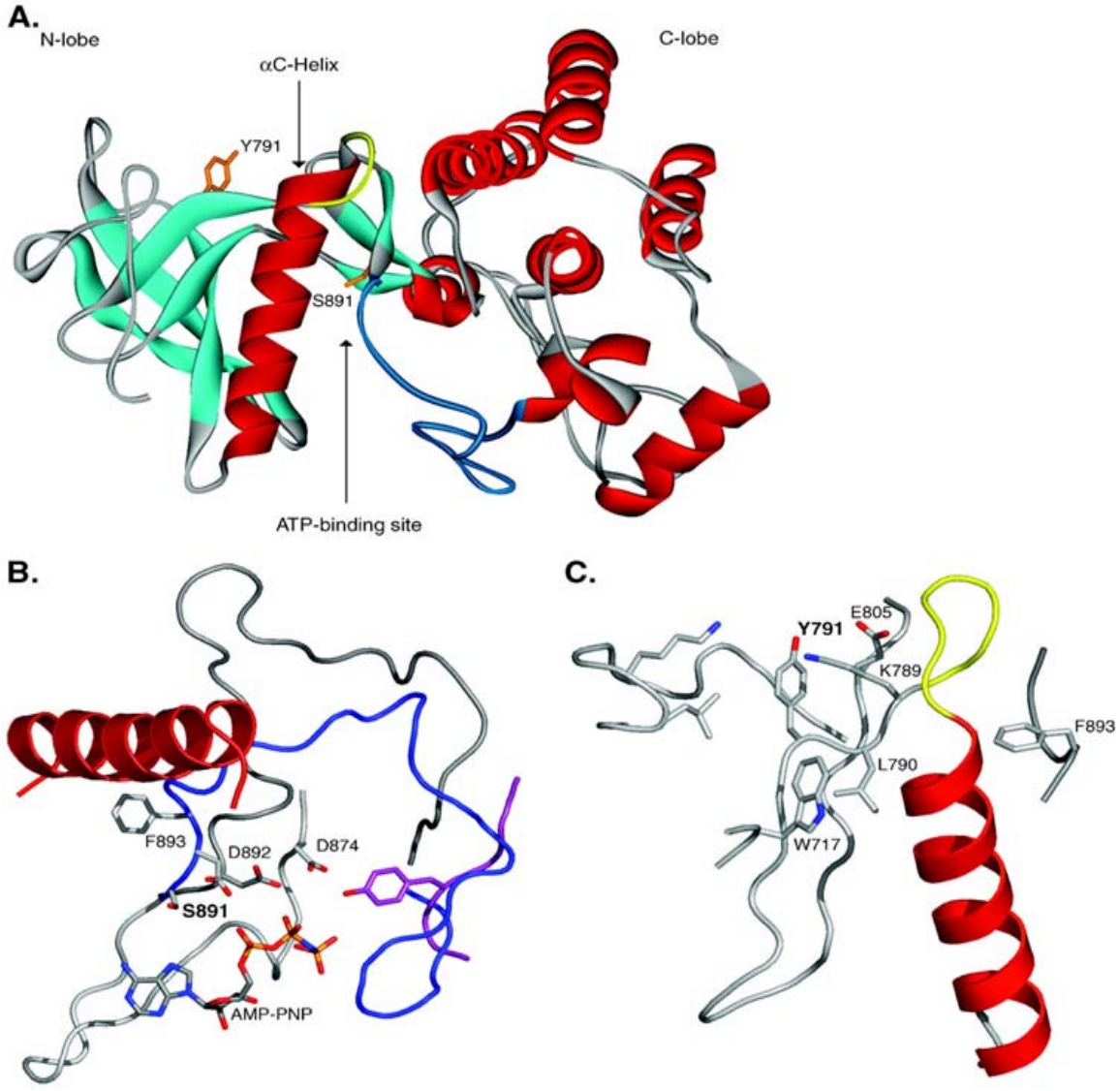

Figure 6

Structural modeling of the RET tyrosine kinase domain affected by mutations Y791F and S891A

Schematic representation of modeled RETK showing the N-terminal lobe at the left hand side and the C-terminal lobe at the right hand side. The activation loop is depicted in dark blue and the loop connecting the αC helix and the β4 strand in yellow (panel A). Activation loop conformation of inactive RETK (dark blue) and activated RETK (dark grey) diverge at residue Ser 891. Activation loop in the inactive conformation blocks access of peptide substrate to the active site and Asp 892 is not properly positioned for interaction with the β-phosphate group of ATP through coordination with Mg2+. Backbone of modeled RETK in light grey, activation loop in blue and αC helix in red. The superimposed activation loop of RETK in active conformation was modeled using activated insulin tyrosine receptor kinase as template structure, ATP analog AMP-PNP and tyrosine peptide substrate (purple) coordinates were also taken from this structure (47) (panel B). Local environment around tyrosine 791: N-terminal lobe backbone residues in light grey and backbone residues of the DFG motive in dark grey, αC helix in red and loop connecting the αC helix and the β4-strand in yellow (panel C).

DISCUSSION

Many groups have investigated how mutations in the *RET* proto-oncogene can result in constitutive active receptors and which substrates and signalling routes are being activated by these aberrant proteins. Finding mutation-specific alterations in RET signalling properties might help us to understand how different *RET* associated cancer syndromes do develop. Nevertheless, the oncogenic activation and downstream signalling pathways activated by FMTC mutations located within the catalytic domain of RET have been just partially uncovered (41, 48).

We now show that RETY791F is active as a monomeric protein and that both RETY791F and RETS891A are phosphorylated and activated independently of GDNF. Results similar to the observations reported for RETS891A (41). Signalling by the monomeric RETY791F and RETS891A proteins is GDNF independent and strongly targets the STAT3 signalling pathway. Although there is evidence that RET induced signalling is cell type specific (24), our results indicate that the activation of STAT3 by both RETY791F and RETS891A mutants was not cell type dependent as four different cell lines (HepG2, HEK293, COS-7, SK-N-SH) gave similar results. Previously it has been reported that the oncogenic transactivation of STAT3 by RETC634R (a mutation found in MEN2A patients) was required for cellular transformation, and this process was mediated by the intrinsic tyrosine kinase domain of RET, independently of JAKs and SRC (17), data we largely confirm (see results section). Here we demonstrate, from the use of either specific inhibitors or dominant negative forms of SRC, JAK1 and JAK2, that SRC and JAKs are implicated in STAT3 activation by RETY791F and RETS891A.

Our results suggest that different disease phenotypes associated RET mutations activate STAT3 through different signalling routes. Nevertheless, it is still unclear if the interaction between c-SRC, JAKs and both RETY791F and RETS891A is promoted by direct phosphorylation through the catalytic domain of the receptor itself, or by another parallel mechanism of signalling. Overexpression of JAK1, JAK2 and c-SRC in combination with both RETY791F and RETS891A in the absence of STAT3 did not increase IRE reporter activity significantly; but expression of v-SRC did enhanced it (data not shown), suggesting that aberrant signaling through STAT3 requires the catalytic domain of the receptor “per se”, as well as it is mediated by a

SRC/JAK dependent mechanism that could not be directly linked to the RET receptor itself.

The oncogenic mechanism of activation promoted by the investigated monomeric FMTC-RET mutations located within the catalytic domain differs from the MEN 2A/FMTC mutations located at the cystein rich motif. The extracellular mutations lead to the formation of covalent dimmers due to intermolecular disulfide bounds between RET monomers. Hence, the receptor is constitutive activated, independently of GDNF (49). FMTC mutations affecting the catalytic domain of RET however seem to have an effect similar to the MEN2B mutations. These mutations result in monomeric oncoproteins, altering both the catalytic activity and substrate specificity of the receptor due to structural changes of the binding pocket of the tyrosine kinase domain. They lead to an aberrant phosphorylation of substrates preferred by cytoplasmic tyrosine kinases such as c-SRC and c-ABL (50). We have seen such an effect for the RETY791F and RETS891A mutant proteins. However, the RETM918T (MEN2B) has been also shown to be GDNF responsive, suggesting that differences in the mechanism of receptor activation combined with differences in GDNF-responsiveness of these receptors, as well as tissue-specific GDNF expression (or related ligands), could give rise to different disease phenotypes (24).

STATs are a family of latent transcription factors that become activated in response to cytokines and growth factors (43). Interferon, G-CSF and cytokines activate STAT3 through their receptors in a JAK dependent manner, whereas growth factor receptors can phosphorylate STAT3 through their intrinsic tyrosine kinase domains (43-44). STAT3 has been implicated in oncogenesis of many types of tumors, such as leukemia, lymphomas, multiple myeloma, breast cancer, and head and neck cancers. In all of them constitutive, ligand independent activation of STAT3 has been detected (51). In this study we show that STAT3 plays an important role in the development of medullary thyroid carcinomas (MTC), not only based on *in vitro* data, but also based on the immunohistochemistry analysis of MTCs. Tumors from patients carrying the *RET* Y791F germline mutation showed a clear nuclear localization of activated STAT3 (Phospho Tyr 705) in contrast to normal cells where STAT3 is not phosphorylated and mainly cytoplasmic localized. Therefore, we hypothesize that aberrant STAT3 activation by both FMTC mutations could promote the disregulation of the transcriptional control of genes playing an important role in tumorgenesis, contributing to the malignant transformation of the c-cells resulting in MTC.

Immunohistochemical analysis of tumor tissues revealed as well high levels of RET protein at the plasma membrane, as it was also observed in MTC derived cell lines MTC-TT and MZ-CRC-1. Interestingly in FMTC tumor tissues cytoplasmic localized RET was also observed. We assume, therefore, that the antibody is also recognizing the immature form of the receptor located in the cytoplasm. It remains unclear if the immature form of RET could also be active inside the E.R. while is being processed and transported to the plasma membrane.

How RET, which normally is activated upon dimerization of two proteins, can be active as a monomer is yet unclear. We therefore modeled the RET catalytic domain using the tyrosine kinase domain of FGFR1K (fibroblast growth factor receptor 1) as a template. Our modeling studies suggest that the mutations result in a modification of the tertiary structure of the catalytic domain giving rise to a protein with a more accessible substrate and ATP conformation.

In summary, these results show that FMTC-RET mutants Y791F and S891A constitutively activate STAT3 *in vitro*, a finding supported by observations in tumor material from FMTC patients. Constitutive ligand-independent signalling of these mutant receptors through STAT3 might contribute to the development of medullary thyroid carcinomas.

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Chapter 3

Oncogenic RET induces STAT3Ser727 phosphorylation via a RAS/RAF/MEK1-2/ERK1-2 pathway

Submitted

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ABSTRACT

Aberrant activation of STAT3 by mutant RET is observed in Multiple Endocrine Neoplasia type 2 (MEN2) associated tumors. This activation is mediated by phosphorylation of Tyr705 which causes STAT3 dimerization and nuclear translocation. In the nucleus, STAT3 transcriptional activity can be enhanced by phosphorylation of Ser727. In this study we show that oncogenic RET, but not wild type RET, induced STAT3-Tyr705 phosphorylation. In addition, oncogenic RET induced constitutive STAT3-Ser727 phosphorylation, whereas RET wild type was only able to induce phosphorylation of Ser727 in a ligand-dependent manner. Using chemical inhibitors, dominant negative constructs and westerns analyses we determined that RET induced STAT3-Ser727 phosphorylation via a pathway involving RAS-RAF-MEK1/2-ERK1/2. We could further show that this STAT3-Ser727 phosphorylation enhanced transcriptional activation of the STAT3 target genes *CYCLIND1, ICAM1 AND BCL-XL*. Moreover, inhibition of the ERK1/2 pathway resulted in decreased phosphorylation of STAT3-Ser727 and inhibited proliferation of a MEN2-derived tumor cell line. Immunohistochemical analyses of MEN2 tumor samples showed strong nuclear staining of phosphorylated ERK1/2 and Ser727 phosphorylated STAT3. These data show that dysregulation of both the STAT3 and RAS/MAPK pathways by oncogenic RET converges on STAT3-Ser727 which could play an important role in the development of MEN2 tumors.

INTRODUCTION

Signalling by the receptor tyrosine kinase RET is crucial for the development of neural crest-derived cells lineages and kidney organogenesis (1). In the presence of GFR-$\alpha_{(1-4)}$ co-receptors, RET is activated by members of the Glial Derived Neurotrophic Factors (GDNF) family (1). Different activating missense mutations in the *RET* proto-oncogene cause Multiple Endocrine Neoplasia type 2 (MEN2), a dominant inherited cancer syndrome affecting several neuroendocrine tissues (1). Three different clinical subtypes can be recognized depending on the affected tissues and mutations found: MEN2A, MEN2B and FMTC (1). The molecular mechanisms connecting the mutated receptors with the different clinical subtypes are largely unknown despite clear phenotype-genotype correlations (2).

In MEN2, aberrant STAT3 activation through Tyr705 phosphorylation by mutated RET receptors has been reported (3-5). STAT3 is a latent transcription factor implicated in several types of cancer when aberrantly activated (6-7). Activation of STAT3 is triggered by phosphorylation of Tyr705 in its SH2 domain, resulting in dimerization and nuclear translocation. STAT3 Tyr705 phosphorylation can be mediated by Janus kinases or by growth factor receptors directly (6, 8-9). Moreover, phosphorylation of STAT3 on Ser727, situated in the C-terminal transactivation domain (TAD), results in enhanced transcriptional activation and DNA binding capacity (9). Various kinases have been shown to phosphorylate STAT3 on Ser727, depending on the cytokines and growth factors involved and the cellular context. Examples are: ERK1/2 (10), MSK1 (11), c-Jun N-terminal kinase (12), p38 (9) and TAK1-Nemo-like kinase (13).

In this study we delineated the signalling pathway connecting the RET receptor to STAT3 Ser727 phosphorylation, its biological consequences in RET-mediated tumor cell proliferation and extended our findings to tumor samples from patients carrying germ line activating *RET* mutations.

MATERIALS AND METHODS

Cell lines and cell culture reagents

HEK293 (human embryonic kidney) and MTC-TT (human thyroid medulla carcinoma) cells were grown as described previously (5), TGW (human neuroblastoma) cells (14) were cultured in RPMI medium containing 10% FCS (Gibco), further supplemented with 100 IU/ml penicillin, 1 mg/ml streptomycin and 2 mM L-glutamine (Invitrogen). To generate stably transfected HEK293 cell lines, 10^6 HEK293 cells were plated in 10 cm dishes and transfected with 1μg of RET wild type, RETY791F or RETS891A expression plasmid. 24 hr after transfection, cells were washed and cultured in medium containing 500 μg/ml of G418 (Sigma). Clones were picked after two weeks and screened for RET expression by western analysis. Cells were serum-starved for 1 hour prior to GDNF stimulation (15 min, 50 ng/ml, Prepotech, Rocky Hill, NJ, USA). AG1296, LY and U0126 (Promega, Madison, USA) were used as indicated in the text.

MTT proliferation assays

MTC-TT cells were plated in quadruplicate in 96-well plates (25,000/well) and treated with U0126 (2, 10 and 40 μM). MTC-TT cell proliferation was followed for five days using an MTT assay according to the manufacturer's (Roche) instructions.

RNA extraction, cDNA synthesis and RT-PCR

The RNAeasy protect mini kit (Qiagen) was used to extract total RNA from HEK293 cell lines. For cDNA synthesis, 200 ng of total RNA was used with ready-to-go-your-prime first strand beads (Amersham Biosciences) using oligo-dT primers as indicated by the manufacturer. For RT-PCR, *FOS* (F 5'-TGCCAACTTCATTCCCAGGGT-3', R 5-TAGTTGGTCTGTCTCCGCTTG-3'), EGR-1 (F 5'-TTTGCCAGGAGCGATGAAC-3', R 5-CCGAAGAGGCCACAACACTT-3') and ACTIN (F 5'-GCTCGTCGTCGACAACGGCT3', R 5-CAAACATGATCTGGGTCATCTTCTC-3') primers were used and PCR reactions were performed under standard conditions.

Expression and reporter plasmids

The expression plasmids pRC-CMV-RETwt (RETWT), RETS891A, RETY791F, pGL5-STAT3α, STAT3S727A and STAT3β were described previously (5). The dominant negative

(dn) RAS, dominant negative RAF, dominant negative SEK1, pUAS-luc, pGAL4-ELK-1, and the full length promoter-luciferase construct of *CYCLIND1, ICAM1* were kindly provided by members of the Department of Hematology (UMCG, Groningen, The Netherlands). The reporter plasmids pTAL-SRE-luc, pIRE-ti-Luc and pDM2-LacZ were described previously (5,15) and the *BCL-XL*-promoter-luciferase construct was kindly provided by the Department of Pulmonary diseases AZU, Utrecht, The Netherlands.

Luciferase reporter assays

HEK293 cells were transfected using a calcium phosphate method as described previously (3, 15). Briefly, 24 hr after transfection, cells were washed and treated with inhibitors (overnight) as indicated, the next day; cells were harvested in lysis buffer (Promega). Luciferase activity was determined using the SteadyLite HTS kit (Perkin Elmer). In all transfections, a β-galactosidase expression plasmid (pDM2LacZ) was included to normalize luciferase activities. β-Galactosidase activity was determined in 100 mM Na_2HPO_4/NaH_2PO_4, 1 mM $MgCl_2$, 100 mM 2-mercaptoethanol and 0.67 mg/ml O-nitrophenylgalactopyranoside.

Western blotting

Cells were lysed with 10 mM Tris-Cl pH 7.4, 144 mM NaCl, 2 mM EDTA, 1% Nonidet P40, 2 mM DTT, 1 mM Na-vanadate, 87% glycerol, 10 μg/ml aprotinin, 2 μg/ml leupeptin, 0.2 mM PMSF. Lysates were resolved on SDS-PAGE and analyzed using western blotting and ECL (Roche). The following antibodies (1:1000) were used: RET (H-300), phospho-Tyr1062RET, STAT3 (C-20) (Santa Cruz), phospho-Tyr981RET (5), phospho-ERK1/2, ERK1/2 and phospho-STAT3 (Tyr705 and Ser727, Cell Signaling Technology, New England Biolabs, UK). For immunoprecipitation, protein A sepharose beads were coated with ERK1/2 antibodies by 1 hour rotation at 4°C. Beads were washed with lysis buffer, and added to whole cell protein extracts and rotated overnight at 4°C.

Immunohistochemistry

Immunohistochemistry was performed on tumor samples of independent patients all carrying germ line FMTC-RET mutations, as described previously (16).

RESULTS

Oncogenic RET induces constitutive STAT3 Tyr705 and Ser727 phosphorylation

The mutants RETY791F and RETS891A (two mutations targeting the tyrosine kinase domain of RET and associated with the FMTC phenotype) signal independent of GDNF and induce STAT3 Tyr705 phosphorylation through a pathway involving Src and JAK (5). To analyze if RETY791F and RETS891A contribute to aberrant transcriptional activity of STAT3, we investigated whether these mutants also induce STAT3 Ser727 phosphorylation. Western analyses of HEK293 cells, transfected with RETWT, RETY791F and RETS891A, indicated that both mutant receptors were activated in the absence of GDNF, as indicated by RET Tyr1062 phosphorylation. These mutants induced STAT3 phosphorylation on Tyr705 and Ser727 (Fig. 1A, left panel). Stimulation of these mutant RET receptors by GDNF did not further enhance STAT3 phosphorylation, neither on Tyr705 (5), nor on Ser727 (data not shown). To further corroborate these findings we analyzed the degree of STAT3 Tyr705 and Ser727 phosphorylation in GDNF-treated human neuroblastoma TGW cells, that express wild type RET and the GFRα1 co-receptor, as well as in a metastatic MTC-derived tumor cell line MTC-TT, that expresses oncogenic RET in which Cys 634 is mutated into an Trp. In TGW cells, stimulation with GDNF (50 ng/ml, 15 min) resulted in activation of RETWT as indicated by high levels of Tyr1062 phosphorylated RET (Fig. 1A central panel). Notably, GDNF stimulation of TGW cells did not result in STAT3 Tyr705 phosphorylation whereas increased levels of STAT3 Ser727 phosphorylation were observed (Fig.1A, central panel).

In MTC-TT cells, stimulation with GDNF did not result in increased RETC634R Tyr1062 phosphorylation and also did not further enhance phosphorylation of STAT3 on Tyr705 and Ser727 (Fig. 1A, right panel). These results indicated that oncogenic RET receptors aberrantly activate STAT3 by phosphorylation of Tyr705 and Ser727.

Next, we investigated the effect of oncogenic RET-induced STAT3 Ser727 phosphorylation on the promoter activity of STAT3 target genes. A STAT3 mutant, STAT3S727A in which Ser 727 is mutated into an Ala, was expressed in HEK293 cells in combination with oncogenic RET constructs and various luciferase reporters. Activation of an IRE-reporter by RETS891A was increased by co-expression of STAT3α whereas expression of STAT3S727A reduced reporter activation (Fig. 1B).

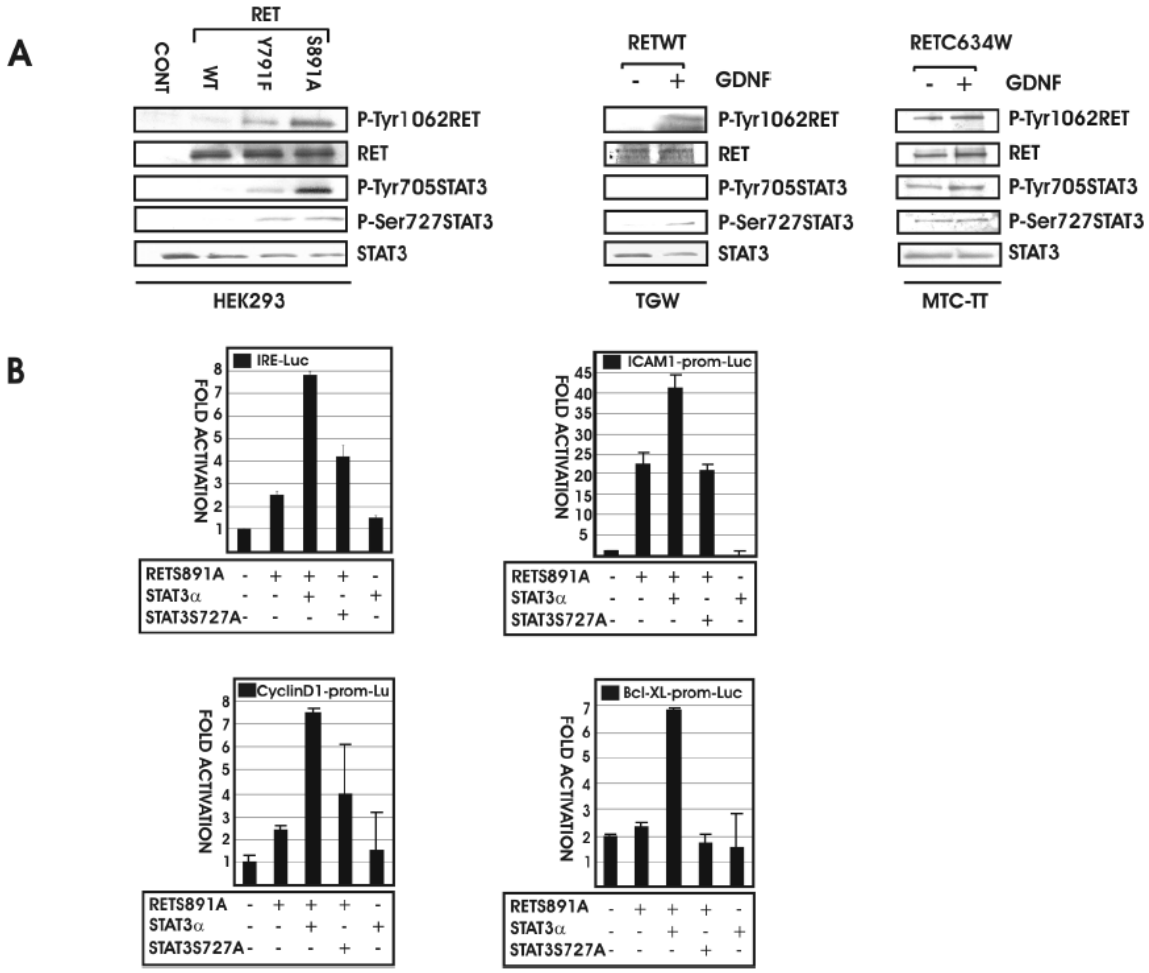


Figure 1

Oncogenic RET induces constitutive activation of the STAT3 pathway

A) HEK293 cells were transfected with RETWT, RETY791F and RETS891A. TGW and MTC-TT cells were stimulated with GDNF (50 ng/ml; 15 min.) as indicated. Cell lysates were western analyzed with phospho-Tyr1062 RET, RET, phospho-Tyr705 STAT3, phospho-Ser727 STAT3 and STAT3 antibodies, as indicated. B) HEK293 cells were transfected with expression plasmids and luciferase reporters as indicated. The average fold activation of normalized luciferase activity with their SD is depicted.

In order to analyze the role of Ser727 phosphorylation in RET-mediated transcriptional activation of STAT3 target genes, reporter assays were performed using the promoter regions of the *CYCLIND1, ICAM1 AND BCL-XL* genes fused to a luciferase reporter. In all promoters used, STAT3α potentates reporter activation by RETS891A whereas co-expression of STAT3S727A resulted in reduced transcriptional activation compared to STAT3α (Fig.1B). From these data we concluded that STAT3 Ser727 phosphorylation contributes to

RET-induced transcriptional activation of STAT3 target genes *CYCLIND1, ICAM1 AND BCL-XL*.

Deregulation of ERK1/2 by oncogenic RET

STAT3 Ser727 can be phosphorylated by ERK1/2 (17) and ERK1/2 can be activated by wild type RET (18). To investigate if FMTC-RET mutants aberrantly activate the ERK1/2 pathway, we transfected HEK293 cells with RETWT, RETY791F and RETS891A, in combination with an SRE-luciferase reporter (serum response element), known to be activated by ELK-1, an established ERK1/2 target. Activation of the SRE reporter by both RET mutants was observed when compared to the negative control and RETWT, a 3- and 5-fold induction by RETY791F and RETS891A, respectively (Fig. 2A, left panel). Next, we used a GAL4/ELK-1 reporter system, in which ELK-1 is fused to the DNA binding domain of GAL4, in combination with an UAS-Luc reporter. Robust activation of the UAS-reporter by both mutants was observed (200- and 300-fold for RETY791F and RETS891A, respectively) when compared to an empty expression plasmid and RETWT-induced reporter activity (70-fold). These results indicate that the RET receptor is able to trigger activation of an ERK1/2 pathway that results in ELK-1 activation (Fig. 2A, right panel). Western analyses of HEK293 cells expressing RETWT, RETY791F and RETS891A showed increased levels of ERK1/2 phosphorylation by both mutants (Fig.2B, left panel). When HEK293 cells that stably express RETWT were stimulated with GDNF (50 ng/ml, 15 min.), phosphorylation of both RET (Tyr1062) and ERK1/2 was observed (Fig. 2B, central panel). To further investigate activation of ERK1/2 by mutant RET receptors; we analyzed the mRNA levels of *FOS* and *EGR-1* genes in HEK293 stably expressing RETY791F and RETS891A using RT-PCR. Up-regulation of both these genes was observed in the mutant cell lines, when compared to control HEK293 and HEK293RETWT cells (Fig. 2B, right panel and data not shown). To complement these experiments, we determined the levels of ERK1/2 phosphorylation in response to GDNF in cell lines expressing either wild type RET (TGW) or MEN2A-RET (MTC-TT). In TGW cells, stimulation with GDNF (50 ng/ml, 15 min.), resulted in RET phosphorylation and activation of ERK1/2 (Fig. 2C, left panel). In MTC-TT cells, RET and ERK1/2 phosphorylation was observed in untreated cells and stimulation with GDNF had no effect (Fig.2C, right panel). Taking these results together, aberrant activation of ERK1/2 by oncogenic RET is observed in MTCs.

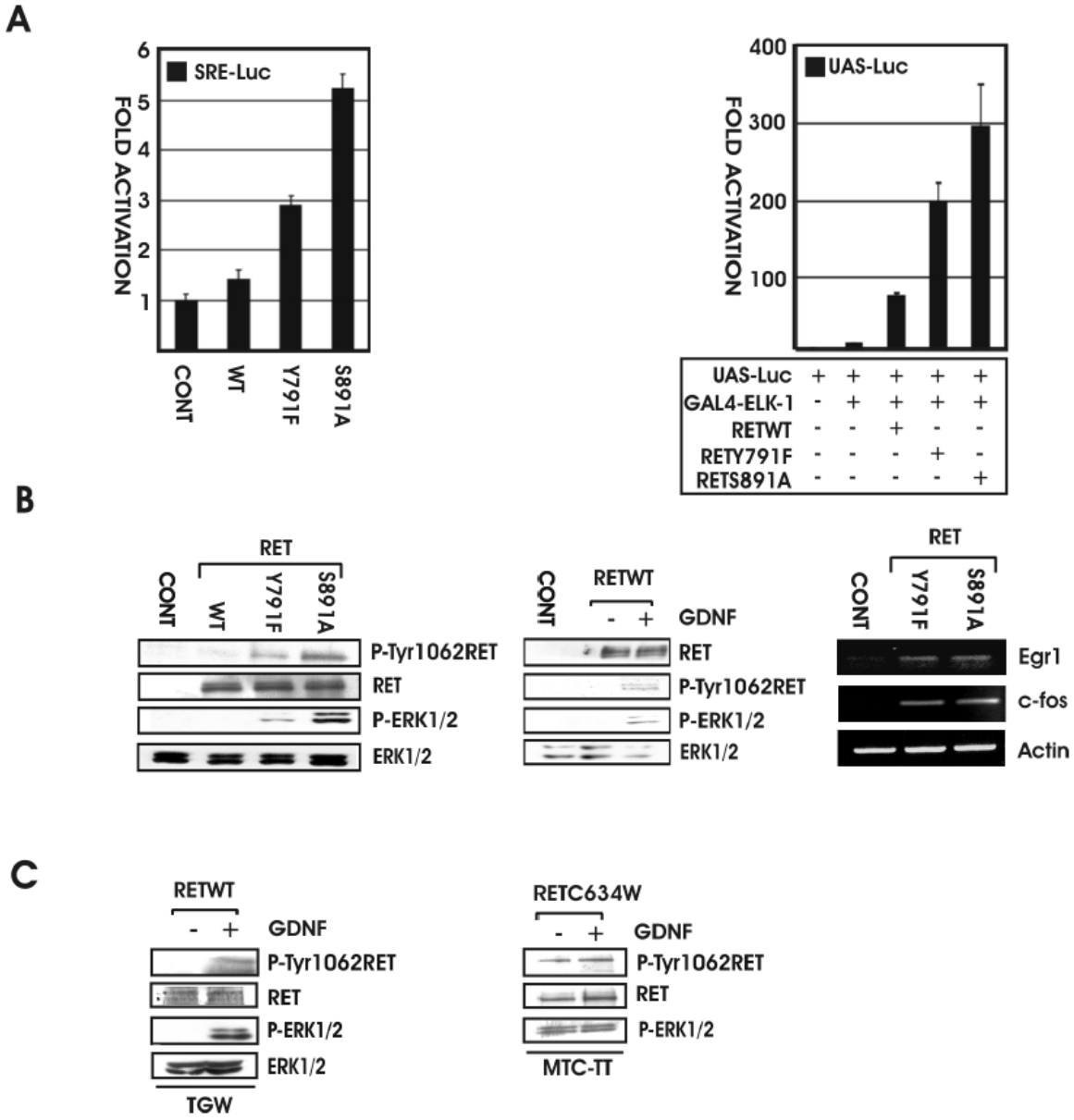


Figure 2

Oncogenic RET activates ERK1/2, in the absence of GDNF

A) HEK293 cells were transfected with expression plasmids and reporters as indicated. The average fold activation of normalized luciferase activity with their SD is depicted. B) HEK293 cells were transfected as indicated and cell lysates were western analyzed using antibodies against: phospho-Tyr1062 RET, RET, phospho-ERK1/2, ERK1/2 and as indicated. HEK293 cells, stably transfected with RETWT, and transiently transfected with GFRα-1, were stimulated with GDNF (50 ng/ml, 10 min) as indicated. RT-PCR on cDNA from HEK293-RETY791F, HEK293-RETS891A, and HEK293 control cells, showing up-regulation of c-*FOS* and *EGR*-1 mRNA levels in mutant cell lines. C) Tumor cell lines TGW and MTC-TT were stimulated with GDNF (50 ng/ml, 15 min) and protein cell extracts were western analyzed with phospho-Tyr1062 RET, RET, phospho-ERK1/2 and ERK1/2 antibodies.

RET induces STAT3 Ser727 phosphorylation via a RAS-RAF-MEK1/2-ERK1/2 pathway

To determine if RET-induced STAT3 Ser727 phosphorylation was mediated through a RAS/ERK1/2 pathway, HEK293 cells expressing RETS891A were treated with the tyrosine kinase receptor inhibitor AG1296. A reduction of RET Tyr981 and Tyr1062 phosphorylation was observed at concentrations of 20 μM AG1296, indicating reduced RET kinase activity (Fig. 3A). A reduction in phosphorylation levels of signaling molecules downstream of RET such us STAT3 on Tyr705 (data not shown and (5)), STAT3 Ser727 and ERK1/2 was observed (Fig. 3A). These results suggest that RET signalling is required for STAT3 Tyr705, STAT3Ser727 and ERK1/2 phosphorylation. Next, GAL4-ELK-1/UAS-Luc reporter assays were used to delineate the pathway from the RET receptor to ERK1/2 activation. Co-expression of either dominant negative (dn)-RAS or dn-RAF resulted in a complete loss of UAS-reporter activation, indicating that RAS and RAF are required for ELK-1 activation by RETY791F and RETS891A (Fig. 3B). In case the IRE-Luc reporter was used, dn-RAS and dn-RAF partially reduced reporter activation by RETS891A where expression of a dn-SEK1 construct (16) had no effect on RETS891A-induced IRE-Luc activity, indicating that RAS and RAF, but not SEK1, are involved in ERK1/2-mediated STAT3 activation. To further confirm the reporter data, we determined the requirement of RAS for ERK1/2 and STAT3 Ser727 phosphorylation in HEK293 cells expressing RETS891A. Expression of dn-RAS in these cells resulted in decreased levels of ERK1/2 and STAT3 Ser727 phosphorylation, respectively (Fig. 3B and C).

To test whether MEK1/2 (a MAPKK upstream of ERK1/2) was activating ERK1/2 in response to RETS891A, we tested the effect of the MEK1/2 inhibitor U0126 on RETS891A-induced UAS-Luc and IRE-Luc activation. U0126 (10-40 μM) reduced both UAS-Luc and IRE-Luc activation by RETS891A by approximately 70% and 50%, respectively (Fig. 3D and E). Western analysis indicated that U0126 was able to completely inhibit RETS891A-induced ERK1/2 and STAT3 Ser727 phosphorylation (Fig. 3F).

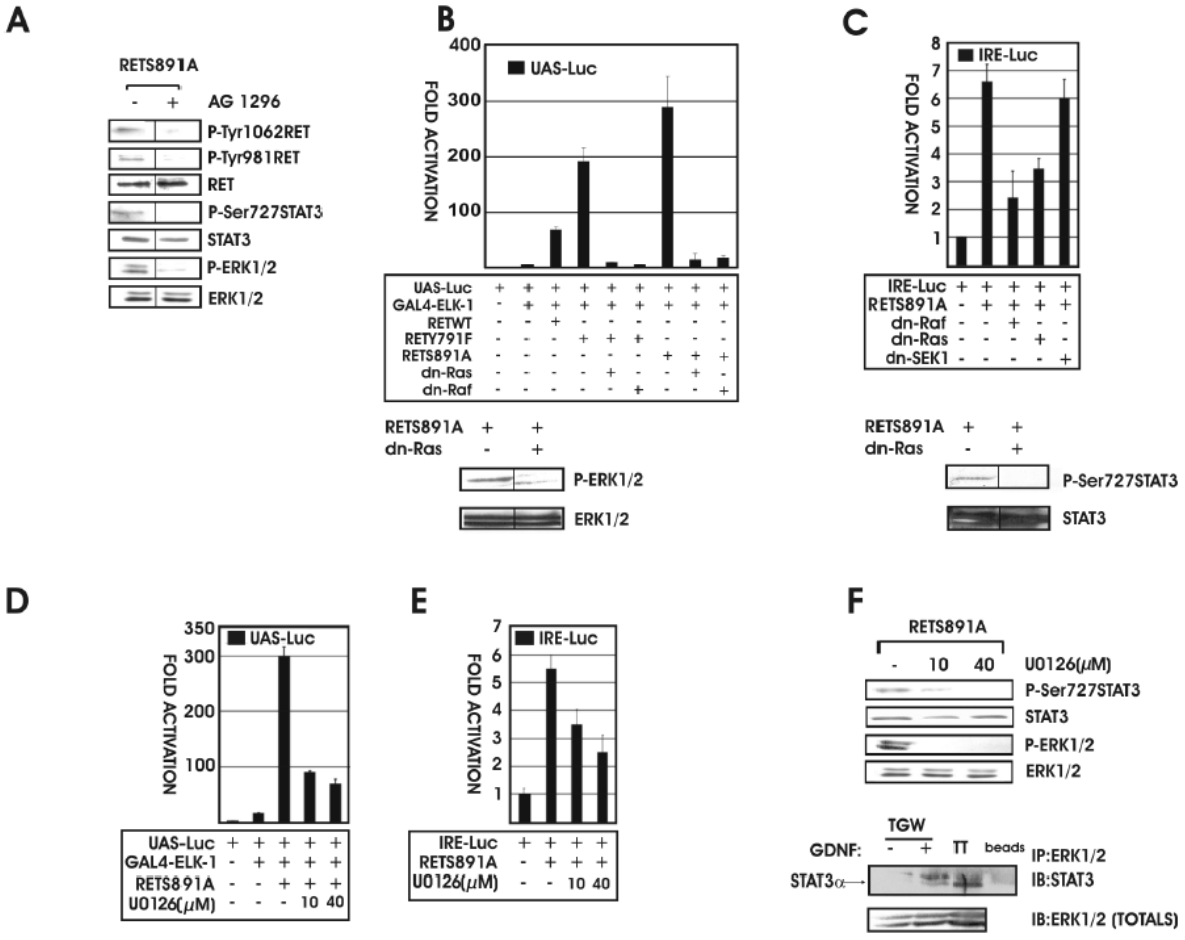


Figure 3

STAT3 Ser727 phosphorylation by a RET/RAS/RAF/MEK1-2/ERK1-2 pathway

A) HEK293 cells were transfected with RETS891A and treated with AG1296 as indicated. Western analyses were performed using antibodies against: phospho-Tyr 1062 RET, phospho-Tyr981 RET, phospho-Ser727 STAT3, STAT3, phospho-ERK1/2 and ERK1/2. Samples (-, 20 μM AG1296) were resolved on the same gel but not in adjacent lanes. B-E) Luciferase reporter assays were performed in HEK293 cells transfected with the indicated expression and reporter plasmids and treated with U0126. The average fold activation of normalized luciferase activity with their SD is depicted. B,C) Western analysis were performed with HEK293 cells transiently expressing RETS891A and dn-RAS as indicated, using phospho-Ser727 STAT3, STAT3, phospho-ERK1/2 and ERK1/2 antibodies, respectively. Samples were resolved on the same gel but not in adjacent lanes. F) HEK293 cells were transfected with RETS891A and treated with U0126 as indicated. Cell extracts were western analyzed with antibodies against phospho-Ser727 STAT3, STAT3, phospho-ERK1/2 and ERK1/2 as indicated. Total cell lysates from TGW, stimulated with GDNF (50 ng/ml, 15 min.), and MTC-TT cells were subjected to immunoprecipitation using ERK1/2 antibodies, and western analyzed with STAT3 antibody (IP: ERK1/1, IB: STAT3). Uncoated beads were used as a control and total cell lysates were probed against ERK1/2 (Totals).

To demonstrate that ERK1/2 and STAT3 form a complex during RET signalling, immunoprecipitation was performed in TGW (-/+ GDNF) and MTC-TT cells. Whole cell lysates were immunoprecipitated using ERK1/2 antibody and western analyzed for STAT3. Complex formation between STAT3 and ERK1/2 was seen in GDNF-stimulated TGW cells and in untreated MTC-TT cells (Fig. 4G). From these results we concluded that RET-induced STAT3 Ser727 phosphorylation was mediated by a RAS/RAF/MEK1-2/ERK1-2 pathway.

Immunohistochemical analysis of tumors samples

Analysis of tumor samples from five patients carrying a *RET* S891A germline mutation was performed. All tumor samples displayed high levels of RET expression at the plasma membrane and in the cytoplasm (Fig. 4A and B). Due to space limitations, representative staining of only one biopsy are shown. A biopsy section containing both normal and tumor tissue was taken as a control. In the area containing normal tissue, highly organized tissue with low levels of RET expression was observed (Fig. 3C). In the area containing the carcinoma, disorganized tissue with high levels of RET expression were detected (Fig. 3C, see arrows). Strong nuclear staining for Ser727 phosphorylated STAT3 was seen in all tumor tissues (Fig. 3D, E). Weak staining was observed for Ser727 phosphorylated STAT3 in areas lacking tumor tissue where strong staining was detected in the area containing tumor tissue (Fig. 3F, see arrows). Finally, strong nuclear and cytoplasmic phospho-ERK1/2 staining was observed in MTC tumor samples (Fig. 3H, I and J, see arrows). In areas where no tumor tissue was present, low levels of phosphorylated ERK were detected (Fig. 3J).

ERK1/2 activity is required for STAT3 Ser727 phosphorylation and MTC-TT cell proliferation

To investigate the biological significance of the interaction between STAT3 and ERK1/2 for RET mediated tumorigenesis, we determined the effect of the MEK1/2 inhibitor U0126 on the proliferation rate of MTC-TT cells (that express RETC634W). Treatment of MTC-TT cells with U0126 (2, 10 and 40 μM) resulted in a dose dependent inhibition of proliferation (Fig. 5A). In parallel, STAT3 Ser727 and ERK1/2 phosphorylation levels were determined in MTC-TT cells treated with U0126 (10 μM, 1 hr) or the PI3K inhibitor LY (20 μM, 1 hr).

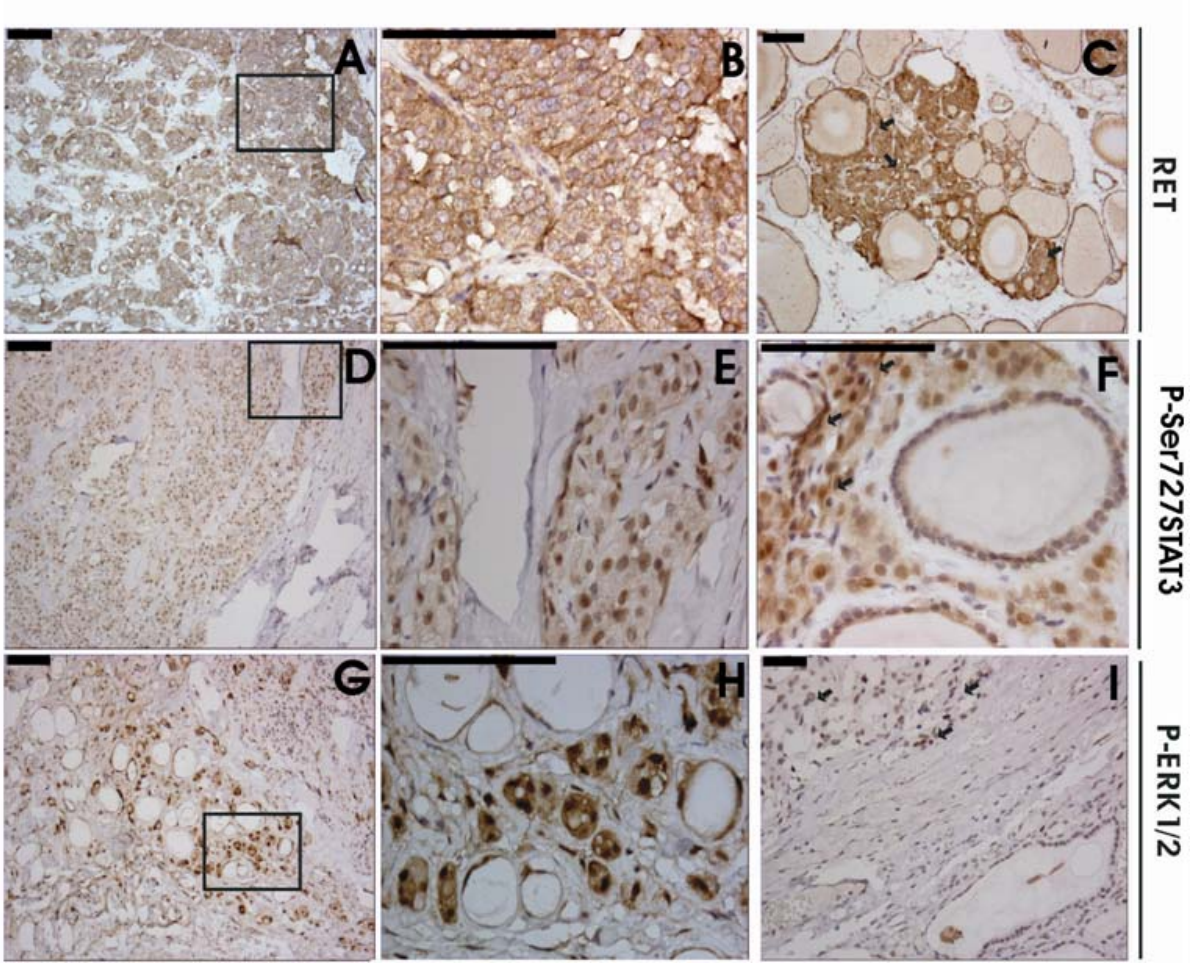


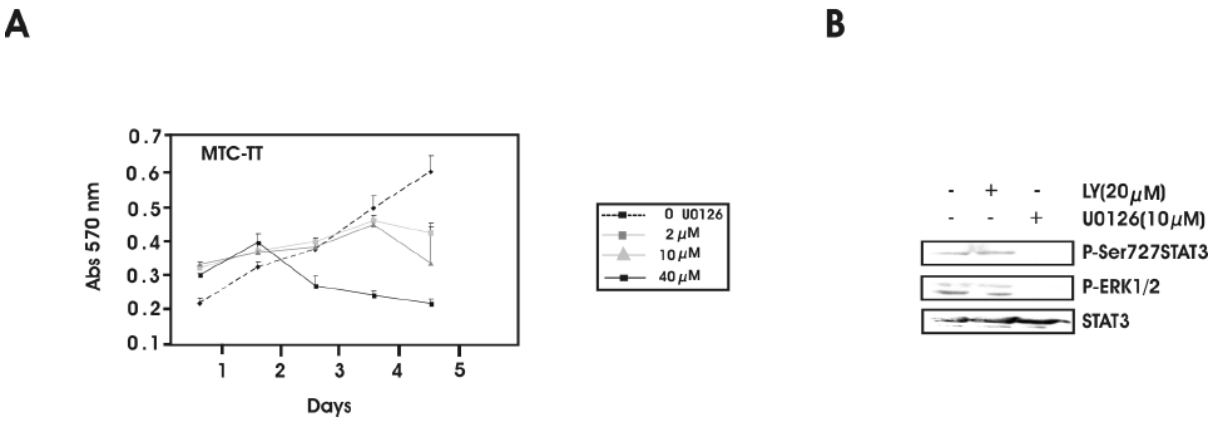
Figure 4

Immunohistochemical analysis of MTC- tumor samples

A,B,D,E,G,H) Medullary Thyroid Carcinoma (MTC) biopsies (100x, left panels), (400x, right panels) from patients carrying a germ line RETS891A mutation stained with anti RET, phospho-Ser727 STAT3 and P-ERK1/2 antibodies as indicated. The frame in the left panels indicates the position of the enlargement shown in the right panels; bar is 100 μm. C, F and I). As controls, sections of MTC biopsies containing both normal and tumor tissue are shown. Higher levels of RET, phospho-Ser727 STAT3 and phospho-ERK1/2, as well as disorganized tissue, was observed in the areas containing the carcinoma (see arrows).

Inhibition of the ERK1/2 pathway by U0126 resulted in a loss of STAT3 Ser 727 phosphorylation, whereas the PI3K inhibitor LY had no effect on ERK1/2 and STAT3 S727 phosphorylation (Fig. 5B).

These data indicate that ERK1/2 is required for STAT3 Ser727 phosphorylation and for proliferation of MTC-TT tumor cells.



ERK1/2 activity is required for STAT3 Ser727 phosphorylation and proliferation of MTC-TT cells

A) MTC-TT cells were treated with U0126 (2, 10 and 40 μM) to determine the requirement of ERK1/2 activity for MTC-TT cell proliferation. A representative experiment is depicted; the average of quadruplicates with the SD is given.

B) Western analysis of MTC-TT cells treated with LY (a PI3K inhibitor) and U0126 (a MEK1/2 inhibitor) with phospho-Ser727 STAT3, STAT3 and ERK1/2 antibodies as indicated.

DISCUSSION

Aberrant activation of STAT3 in MEN2 was shown for MEN2A- (3), MEN2B- (4) and FMTC-associated (5) mutations in the RET proto-oncogene. Activation of STAT3 is triggered by phosphorylation of Tyr705 localized in an SH2 domain (8), which causes STAT3 dimerization and translocation to the nucleus. Once in the nucleus, transcriptional activation of STAT3 can be enhanced by phosphorylation of Ser727, located in the carboxy-terminal transcriptional activation domain (10-12). Whether STAT3 Ser727 phosphorylation was induced by FMTC-RET mutants and if STAT Ser727 phosphorylation contributes to aberrant STAT3 transactivity in MEN2 was unknown and has been addressed in this study. First, we show that oncogenic RET is able to induce STAT3 phosphorylation at both Tyr705 and Ser727, independent of GDNF. However, STAT3 Tyr705 phosphorylation was not induced by GDNF-activated wt RET receptors, whereas phosphorylation of STAT3 on Ser727 was observed. These results suggest that STAT3 Tyr705 phosphorylation is specifically associated with oncogenic RET. Moreover, assays using the IRE-luciferase reporter and the promoter regions of STAT3 target genes *CYCLIND1, BCL-XL* and *ICAM-1* as STAT3 responsive reporters demonstrated that mutation of STAT3 Ser727 to Ala reduced the levels of RET-mediated STAT3 transcriptional activity of these targets genes (Fig. 1B).

Secondly, we demonstrated that oncogenic RET is also able to constitutively activate the ERK1/2-MAPK pathway as SRE- and Elk1/UAS-luciferase reporter activation was induced by RETY791F and RETS891A, in the absence of GDNF. Western blotting and RT-PCR analysis corroborated the reporter data, showing increased levels of phosphorylated ERK1/2 as well as induction of the ELK1 targets genes *FOS* and *EGR1*. Levels of ERK1/2 phosphorylation and SRE and ELK1 reporter activation displayed by RETS891A were higher than RETY791F. These results correlated with the levels of receptor activation and the degrees of STAT3 activation observed (Fig. 1A). These data suggest that the S891A mutant has a higher capacity to trigger proliferative signals. Transformation assays performed in 3T3-NIH cells indeed showed higher transforming capacity of RETS891A then of RETY791, and confirmed the potential oncogenic properties of RETY791F (data not shown).

We were able to delineate a RET-mediated signalling pathway promoting STAT3 Ser727 phosphorylation, involving RAS, RAF, MEK1/2 and ERK1/2. These finding were obtained using dominant negative (dn) intermediates of the RAS/MAPK signalling pathway such as dn-RAS and dn-RAF, as well as with the MEK1/2 inhibitor U0126 in luciferase reporter

experiments, immunoprecipitation and western analyses. Expression of dn-RAS (RasN17) and dn-RAF abolished RETY791F- and RETS891A-induced activation of the UAS-luciferase reporter by GAL4-ELK-1and decreased the phosphorylation levels of ERK1/2 (Fig. 3B). Activation of the IRE reporter by RETS891A and the STAT3 Ser727 phosphorylation levels were reduced by dn-RAS (Fig. 3B), dn-RAF and U0126, indicating that the ERK1/2 pathway is playing a role in the regulation of the RET-STAT3 signaling pathway. The fact that the IRE reporter was not completely blocked by U0126 at concentrations where the SRE and ELK1 reporters were almost completely inhibited is most likely explained by the fact that STAT3-Ser727 phosphorylation potentates transcriptional activation by STAT3 but is not absolutely required for STAT3 activity. The same results are obtained by expression of a STAT3S727A mutant, which is less transactive than wild type STAT3, but still acts as a transcriptional activator (Fig. 1B). A STAT3 splice variant, STAT3β, that lacks the C-terminal transactivation domain, behaves more like a dn-STAT3 in our studies, as overexpression of this construct could reduce levels of IRE reporter activation by RETS891A (5). Immunoprecipitation and western analyses confirmed our reporter data as we were able to demonstrate GDNF-induced complex formation between ERK1/2 and STAT3 (Fig. 3F) as well as functional association between ERK1/2 and STAT3, as U0126 completely abrogated RET-induced ERK1/2 phosphorylation and reduced the levels of RET-induced STAT3 Ser727 phosphorylation (Fig. 3F). However, a complete inhibition of RET-induced ERK1/2 phosphorylation was obtained at 10 μM U0126 where at this concentration; a partial inhibition of STAT3 Ser727 phosphorylation was observed (Fig. 3F). This result suggests that other, non-MEK1/2-ERK1/2 pathways might be involved in mediating oncogenic RET induced STAT3 Ser727 phosphorylation.

We have tried to investigate such alternatives pathways using the dn-SEK1 (19) in our luciferase reporter experiments (Fig. 3C) and the PI3K inhibitor (LY) in western analysis (Fig. 5B), which indicated that these pathways are not involved in RET-induced STAT3 Ser 727 phosphorylation.

Immunohistochemical analyses of tumor samples from patients carrying a germ line RET S891A mutation supported our *in vitro* data since high levels of RET expression at the plasma membrane was observed in combination with strong nuclear staining of both phospho-Ser727 STAT3 and phospho-ERK1/2. These data, in combination with previous

studies (5), suggest that activation of both the ERK1/2 and STAT3 pathways occurs *in vivo* in MTCs.

To obtain some insight in the biological consequence of STAT3 Ser727 phosphorylation, we performed proliferation assays using MTC-TT cells (expressing MEN2A mutant RETC634W), treated with increasing amounts of the MEK1/2 inhibitor U0126. Inhibition of ERK1/2 resulted in both a loss of STAT3 Ser727 phosphorylation and a dose dependent inhibition of MTC-TT cell proliferation (Fig.5).

In conclusion, our data show that aberrant activation of STAT3 by oncogenic RET involves not only the constitutive activation and nuclear translocation of STAT3 by Tyr705 phosphorylation but that these mutants further enhance the transcriptional activity of STAT3 by phosphorylation of Ser727 through a RAS-RAF-MEK1/2-ERK1/2 pathway (Fig. 6). These data suggest that dysregulation of both the STAT3 and the Ras/MAPK pathways by oncogenic RET converge on STAT3 at Ser727, which could play an important role in the development of Medullary Thyroid Carcinomas and gives new insights in the signalling networks causing this type of cancer.

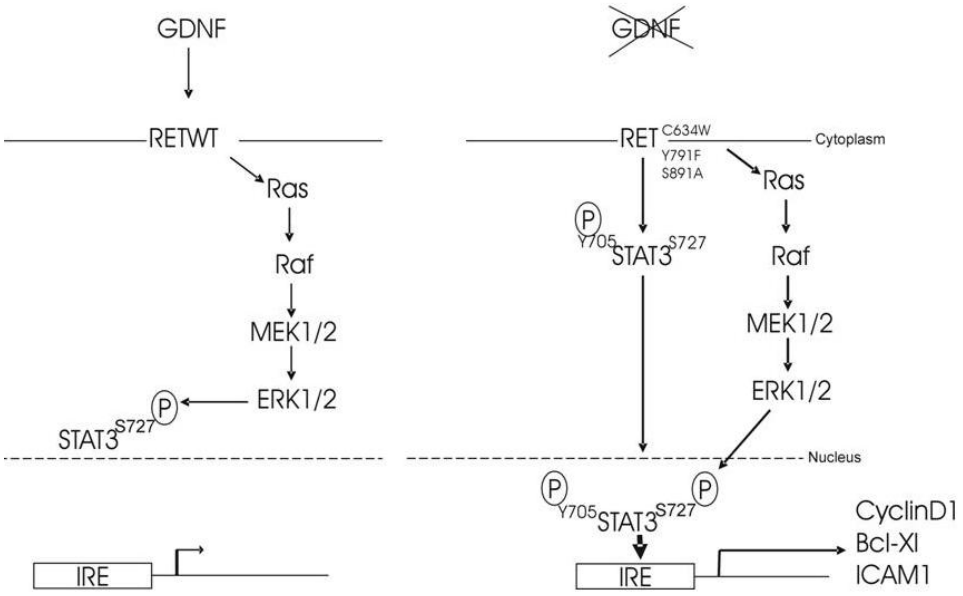


Figure 6

A model for constitutive oncogenic STAT3 activation by RET

Oncogenic RET induces Tyr705 STAT3 phosphorylation, STAT3 transcriptional activity is further enhanced by Ser727 phosphorylation via a RAS/ERK1/2 pathway. In contrast, wild type RET does not trigger activation of STAT3 by Tyr705 phosphorylation, but does, in response to GDNF, promote Ser727 STAT3 phosphorylation by the RAS/MAPK pathway.

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Chapter 4

Differential activation of ERK1/2, STAT3 and SRC by specific Multiple Endocrine Neoplasia type 2 associated RET mutants

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ABSTRACT

The *RET* proto-oncogene encodes a receptor tyrosine kinase whose dysfunction plays a crucial role in the development of several neural crest-related disorders. Distinct activating RET mutations cause Multiple Endocrine Neoplasia type 2A (MEN2A), type 2B (MEN2B) and Familial Medullary Thyroid Carcinoma (FMTC), respectively. Despite clear genotype-phenotype correlations, the molecular mechanisms connecting the mutated receptors with the distinct clinical subtypes are far from understood. Site-directed mutagenesis, luciferase reporter assays, western analysis in combination with structural modelling studies were performed in order to find RET mutant specific signalling. We show that ERK1/2 and STAT3 are differentially activated by specific MEN2-RET oncoproteins and that the activation level of both pathways strongly correlated with the degree of RET Tyr1062 and Tyr981 phosphorylation, respectively. Moreover, phosphorylation levels of RET Tyr1062 and Tyr918 were up-regulated by co-expression of a constitutive active SRC (v-SRC), suggesting *in trans* phosphorylation/activation of RET by v-SRC. Receptor activation was stronger for those intracellular point mutations situated in the proximity of or within the WMxxEx motif, also known as the P+1 loop of RET. Furthermore, we modelled the tyrosine kinase domain of RET with MEN2B mutations, RETA883F and RETM918T. Our modelling results confirmed that MEN2B-type mutations could alter the conformation of the RET kinase domain. In particular, the RETM918T mutation display an increased P+1 and P+3 binding pocket, changing the overall shape of the substrate binding site, possibly altering its substrate specificity. Mutation RETA883F was able to induce both, conformational changes in the activation loop resulting in a more open ATP and substrate binding conformation as well as an alteration in the conformation of the catalytic loop thereby shifting the equilibrium from the inactive to the active state of the kinase domain of RET.

INTRODUCTION

Ligand regulated signalling by the receptor tyrosine kinase RET is crucial for the development of neural crest-derived lineages and kidney organogenesis (1). Dysfunction of RET, on the other hand, plays an important role in the development of several neural crest-related disorders (1). Specific germline missense mutations in the *RET* proto-oncogene that result in constitutive activation of the receptor cause the dominantly inherited cancer syndrome Multiple Endocrine Neoplasia type 2 (MEN2). Depending on the tissues affected and on the mutations found, three different clinical subtypes of MEN2 are being recognized. MEN2A is characterized by Medullary Thyroid Carcinoma (MTC), pheochromocytoma and hyperplasia of the parathyroid. MEN2B is characterized by MTC, pheochromocytoma, but instead of hyperplasia of the parathyroid, patients develop neuromas on the tongue, lips and eyelids, and also intestinal ganglioneuromas. In FMTC, only the c-cells of the thyroid become malignant (1).

Mutations located in the cystein-rich domain of RET give rise to MEN2A and FMTC. Distinct mutations in the tyrosine kinase domain of the receptor can give rise to FMTC or to MEN2B (1). Interestingly, some mutations in the cystein-rich domain are not only found in families with MEN2A/FMTC but also in patients with Hirschsprung's disease (HSCR), a congenital malformation characterized by an absence of enteric ganglia in the distal part of the colon, or in patients having a combination of both MEN2A and HSCR (2).

Despite clear phenotype-genotype correlations, the molecular mechanisms connecting the mutant receptor with the different clinical subtypes are largely unknown, (3). Different oncogenic mechanisms of activation and patterns of receptor auto-phosphorylation have been shown for the different RET-MEN2A and RET-MEN2B oncoproteins (4, 5). As consequence, different sets of phosphotyrosine-mediated signalling pathways might be activated, resulting in a different tissue-specific pattern of gene expression. In this article we studied the degree of activation of ERK1/2, STAT3 and SRC promoted by specific disease phenotype associated RET mutants in order to obtain new insights into the aberrant signalling properties of different MEN2-mutated RET receptors.

EXPERIMENTAL PROCEDURES

Cell lines and cell culture reagents

HEK293 human embryonic kidney cells were grown in Dulbecco´s Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Calf Serum (FCS) (Gibco), 100 μg/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine.

Expression and reporter plasmids

The pRC-CMV-RETwt (RETWT) plasmid encoding the short form of the human *RET* proto-oncogene was used to create RETC620R, RETC634R, RETY791F, RETS891A, RETA883F and RETM918T by site-directed mutagenesis according to the manufacturer's instructions (QuickChange site-directed mutagenesis kit, Stratagene, La Jolla, USA) using the following forward (F) and reverse (R) primers:

RETC620R F 5'- GGCACCTGCAACTACTTCCCTGAGGAGG -3'

RETC620R R 5'- CCTCCTCAGGGAAGTAGTTGCAGGTGCC -3'

RETC634R F 5'- GTGCGACGAGCTGCGCCGCACGGTGATCG -3'

RETC634R R 5'- CGATCACCGTGCGGCGCAGCTCGTCGCAC -3'

RETY791F F 5'-CCACATGTCATCAAATTGTTTGGGGCCTGCAGCCA
GCATGGCCC -3

RETY791F R 5'-GGTGTACAGTAGTTTAACAAACCCCGGACGTCGG
TCGTACCGGG -3'

RETS891A F 5'- GGAAGATGAAGATTGCGGATTTCGGCTTGTCCC -3'

RETS891A R 5'- GGGACAAGCCGAAATCCGCAATCTTCATCTTCC -3'

RETA883F F 5'- GCCAGAAACATCCTGGTATTTGAGGGGCGGA -3'

RETA883F R 5'- CATCTTCCGCCCCTCAAATACCAGGATGTTT -3'

RETM918T F 5'- CGGATTCCAGTTAAATGGACGGCAATTGAAT -3'

RETM918T R 5'- GGGATTCAATTGCCGTCCATTTAACTGGAAT -3'

Following mutagenesis, the entire RET cDNA was checked by sequencing. The pTAL-SRE-Luc, pIRE-Luc and pDM2-LacZ reporter plasmids were described previously (6). The v-SRC plasmid was kindly provided by Dr. J.J. Schuringa (Department of Hematology, UMCG, The Netherlands).

Luciferase reporter assays

HEK293 cells were transfected using the calcium phosphate method as described previously (7). Briefly, HEK293 cells were seeded in 6 well plates (250.000/well) and transfected the next day. After transfection (24 hr), cells were washed and new medium was added. The next day (48 hr) cells were harvested in lysis buffer (Promega). Luciferase activity was determined using the SteadyLite HTS kit (Perkin Elmer). In all transfections, a β-galactosidase expression plasmid (pDM2LacZ) was included as an internal standard to normalize luciferase activities. β-galactosidase activity was determined in 100 mM Na_2HPO_4/NaH_2PO_4, 1 mM $MgCl_2$, 100 mM 2β-mercaptoethanol and 0.67 mg/ml *O*-nitrophenylgalactopyranoside.

Western blotting

Cells were lysed with 10 mM Tris-Cl pH 7.4, 144 mM NaCl, 2 mM EDTA, 1% Nonidet P40, 2 mM DTT, 1 mM Na-vanadate, 10% glycerol, 10 μg/ml aprotinin, 2 μg/ml leupeptin, 0.2 mM PMSF, resolved on a 10% SDS-PAGE and analyzed using western blotting and ECL (Roche). The following antibodies (1:1000) were used: RET (H-300), phospho-Tyr1062RET, STAT3 (C-20) (all of them from Santa Cruz), phospho-Tyr981RET (8), phospho-ERK1/2, ERK1/2 and phospho-STAT3 (Tyr705 and Ser727) (all of them from Cell Signalling Technology, New England Biolabs, UK). Quantification of signal intensity was performed using ImageJ software.

Structural modelling analysis

Structure and sequence databases (PDB and Swissprot) were searched for sequences related to the human RET tyrosine kinase (RETK) domain (Swissprot accession code P07949) using BLAST. Related sequences were aligned with ClustalW using default parameters. The tyrosine kinase domains of human fibroblast growth factor receptor 1 (FGFR1K) and FGFR2K were found to have sequence identities of 50% with RETK. The crystal structure of FGFR1K (PDB accession code 1FGK) determined to 2.0 Å resolution was selected as template to construct the model. The crystal structure of the activated insulin receptor tyrosine kinase domain (PDB accession code 1IR3) was used as template to construct a model of activated RETK. The homology models of inhibitory and activated wild-type RET

and the RETA883F and RETM918T mutants were constructed using MODELLER version 7 as implemented in DS Modelling 1.1 (Acers Inc, San Diego, CA) using standard settings. The kinase insert region of RETK was not included in the final models. The quality of the models was evaluated using Whatif and Procheck. Quality parameters of the models were comparable to those of the template structure. A short energy minimization using constraints on the backbone atoms was performed using the CHARMm module of DS Modeling 1.1.

RESULTS AND DISCUSSION

Activation of RET by GDNF causes trans-phosphorylation of intracellular tyrosine residues triggering downstream signalling pathways required for the development of neural crest derived tissues (such us C-cells of the thyroid gland and enteric neurons) and kidney organogenesis (1). The RET receptor has sixteen tyrosines residues in the intracellular domain, including six tyrosines in the kinase domain (Tyr 864, Tyr 900, Tyr 905, Tyr 928, Tyr 952 and Tyr 981). From these, Tyr 905 has been shown to play a crucial role in the catalytic and cell transforming activity of RET (9) and Tyr981 has been demonstrated to be the docking site for c-SRC and to promote neural survival (8). In the c-terminal tail of RET, Tyr1015 was identified as the docking site for PLC-γ and Tyr1062 as a docking site for SHC/ENIGMA/IRS1/DOK4/5 amongst others (1). Therefore, Tyr1062 seems to be one of the most important docking sites of RET, as mutation of this residue abrogates the RET-mediated activation of the MAPKs (ERK1/2, JNK, P38, ERK5) and PI3-K signalling pathways as well as it able to abrogate the transforming capacity of RET-MEN2A and RET-MEN2B mutants (10). Salvatore and colleagues (11) showed that RETM918T is more active than RETC634R in associating with SHC and that the RETM918T mutation specifically potentiates the ability of RET to auto-phosphorylate Tyr1062 and consequently to trigger higher activation levels of the RAS/MAPK and the PI3K/AKT pathways, respectively (11). In this study we compared not only the most common RET mutations found associated with MEN2A (RETC634R) or MEN2B (RETM918T), but also others disease-phenotype associated RET mutants (Table 1) in order to get a better insight in the molecular mechanism by which the different mutations in the *RET* proto-oncogene give rise different signalling properties and possibly thereby contributing to the different disease phenotypes.

HEK293 cells expressing either RET wild type or a series of MEN2-mutated RET receptors were analyzed by western blotting using an antibody against RET phospho Tyr 1062. The highest level of phosphorylated Tyr1062 was observed with the M918T mutant (MEN2B) (11). Interestingly, RETS891A (a mutation affecting the tyrosine kinase domain and associated with FMTC) showed higher levels of Tyr 1062 phosphorylation than RETC634R and other MEN2B-associated mutation (A883F). RET mutants C620R (MEN2A/FMTC) and Y791F (FMTC) showed increased levels of phosphorylated compared to the wild type receptor but lower than the previously mentioned mutants (Fig. 1A)

Table1.

Genotype-phenotype correlation of the mutations used in the functional studies.

*CRD (cystein rich domain), TKD (tyrosine kinase domain) and CTD (C-terminal tail).

Mutation	Bases substitution	Amino acid change	Exon	Phenotype	Domain*
C620R	TGC to CGC	Cys to Arg	10	MEN 2A	CRD
C634R	TGC to CGC	Cys to Arg	11	MEN 2A	CRD
Y791F	TAT to TTT	Tyr to Phe	13	FMTC	TKD
S891A	TCG to GCG	Ser to Ala	15	FMTC	TKD
A883F	GCT to TTT	Ala to Phe	15	MEN 2B	TKD
M918T	ATG to CTG	Met to Thr	16	MEN 2B	TKD

Next, we tested the levels of ERK1/2 phosphorylation induced by these mutant RET receptors. A similar pattern of ERK1/2 activation, as seen for RET Tyr1062 phosphorylation, was observed. These results suggest a strong correlation between the ERK1/2 pathway and RET Tyr1062 in MEN2 (Fig. 1A). Remarkably, the high levels of RET Tyr1062 phosphorylation displayed by RETS891A did not correlate with the levels of ERK1/2 activity. This could possibly mean that RETS891A, through Tyr1062, interacts with other signalling pathways with a higher affinity than with ERK1/2. Reporter assays in HEK293 cells, in which mutant RET receptors were coexpressed with a GAL4-ELK fusion/UAS-luciferase reporter system, further supported these findings (Fig. 1B).

We also analysed the levels of Tyr981 phosphorylation induced by specific MEN2-RET mutant receptors. RET Tyr981 was reported to be the docking site for c-SRC, and it was shown that a mutation of this residue reduced neural survival promoted by RET (8). Higher levels of phosphorylated RET Tyr981 were shown for RET mutants S891A, M918T and A883F, indicating higher levels of SRC activation for intracellular mutations located in the proximity of or within the WMxxEx motif, also known as the P+1 loop (Fig. 2A, 12). This loop represents a small motif immediately C-terminal of the activation loop. It plays an important role in recognising the residues flanking the target tyrosine in the substrate (12).

Extracellular mutants RET C620R and C634R, as well as RETY791F, a mutation in the β-4 strand of the N-terminal lobe, distantly situated from the P+1 loop, showed lower levels of phosphorylated RET Tyr981, suggesting a lower SRC-dependency of the oncogenic signalling mechanism of the Y791F mutant compared with the S891A mutant (6). Next, we wanted to investigate the levels of STAT3 (one of the main targets of SRC kinase) activation (Tyr705 phosphorylation) promoted by different MEN2-RET mutants. A comparable pattern of STAT3 Tyr705 phosphorylation, as seen for RET Tyr981, induced by different specific RET oncoproteins was observed (Fig. 1A). These results corroborate a previous report in which the RETM918T triggered higher levels of Tyr705 STAT3 phosphorylation than RETC634R (12). Luciferase reporter assays in HEK293 expressing mutated RET receptors with the pIRE-Luc reporter supported these results (Fig.1C). As predicted by the Westerns analyses, higher levels of STAT3 reporter activation were observed by intracellular point mutations M918T, A883F and S891A, followed (in decreasing order) by C634R, Y791F and C620R, respectively (Fig.1C). These results indicate an interaction and signalling cooperation between RET and SRC in STAT3 activation. Some studies indeed have addressed this cooperation between members of the SRC family of kinases and the receptor tyrosine kinase RET (6, 8, 9).

In light of this, we studied the levels of RET phosphorylation *in trans* by co-expression of v-SRC (a constitutive active Src kinase) in HEK293 cells expressing MEN2-associated RET mutations (Fig. 1A). Up-regulation of RET phosphorylation on Tyr981 and Tyr1062 was observed in cells co-expressing RET mutants. Interestingly, the levels of phosphorylated RET were much higher in the case of intracellular point mutants proximal to or within the P+1 loop of the kinase domain of RET (S891A, A883F and M918T) (Fig. 1A). These results suggest that phosphorylation of RET by SRC kinase *in trans* could be an oncogenic mechanism of activation specific for those intracellular point mutations situated in the proximity or within the P+1 loop.

Taken all together, these results shown that signaling differences between different MEN2-associated RET mutations do exist and that these differences most likely will contribute to the clinical differences found associated with the distinct MEN2 subtypes. A table resuming the genotype-signalling-phenotype correlation is depicted in table 2.

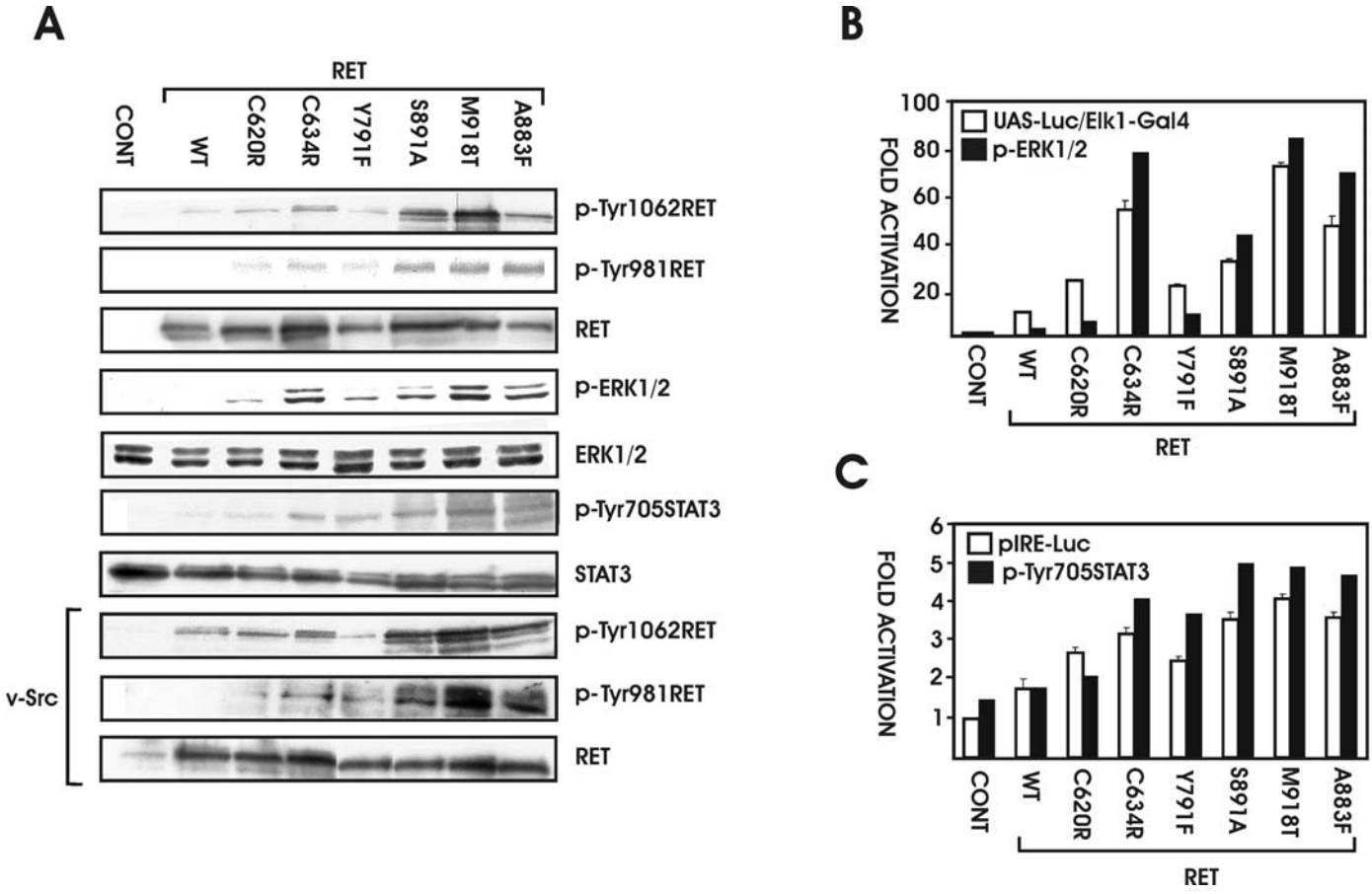


Figure1

ERK1/2, STAT3 and SRC signalling profile by specific MEN 2 disease phenotype associated RET mutants

A) HEK293 cells were transfected with wild type RET or various RET mutants, in combination with v-SRC as indicated. Whole cell protein lysates were resolved (20 μg/lane) on SDS-PAGE and analyzed by Western blotting using antibodies against: phospho-Tyr1062RET, phospho-Tyr981RET, RET phospho-Tyr705STAT3, STAT3, phospho-ERK1/2 and ERK1/2 as depicted.

B) HEK293 cells were transfected with wild type RET and mutant RET receptors in combination with ELK-1/GAL4, UAS-Luc and pDM2-LacZ reporter plasmids. The mean fold activation of the luciferase reporter with the SD of two independent experiments, each of them performed in triplicate, is depicted. Quantification of ERK1/2 phosphorylation was performed using ImageJ software. The data represent the analysis of two independent experiments, scale (1/2).

C) HEK293 cells were transfected with wild type RET and mutant RET receptors in combination with the IRE-Luc and pDM2-LacZ reporter plasmids. The mean fold activation of the luciferase reporter with the SD of three independent experiments, each of them performed in triplicate, is depicted. Quantification of Tyr705 STAT3 phosphorylation was performed using ImageJ software. The data represent the analysis of three independent experiments, scale (1/20).

Table 2

Summary of the signaling properties of MEN2-associated RET mutants.

Mutation	Phenotype	pTyr1062RET	pTyr981RET	pERK1/2	pTyr705STAT3
C620R	MEN2A/FMTC/ HSCR	+	+	+	+
C634R	MEN2A	++	+	+++	++
Y791F	FMTC	+	+	+	+
S891A	FMTC	++	+++	++	+++
M918T	MEN2B	+++	+++	+++	+++
A883F	MEN2B	++	+++	++	+++

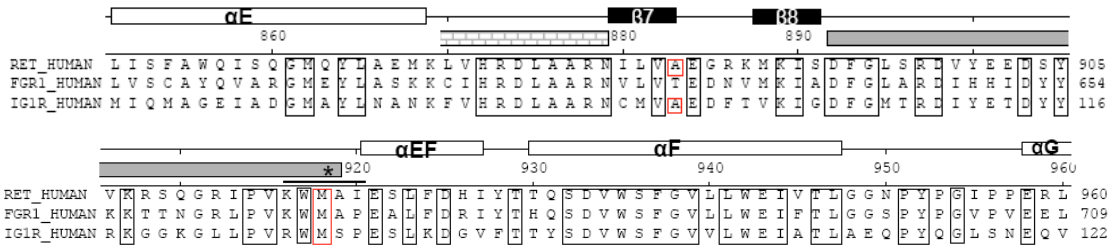
We previously modelled the RET kinase domain (RETK) using the already solved crystal structure of the fibroblast growth factor receptor 1 and 2 tyrosine kinase domains (FGFR1 and 2) as a template (6). To model the conformational changes in the activation loop caused by MEN2B mutations as well as the consequence of these in the receptor's peptide substrate binding properties, we used the crystal structure of the Insulin receptor kinase (IRK) domain as a template (13). Although the percentage in sequence identity between RETK and IRK is smaller (~38%) compared to FGFRK1 (~50%), the overall structural characteristics of receptor tyrosine kinase domains are highly conserved (Fig. 2A), allowing us to use the crystal structure of the activated IRK (13) in complex with a substrate peptide to construct an activated conformation of the RETK model (13). The residues creating the P+1 and P+3 substrate binding pockets are highly conserved between IRK and RETK. The P+1 binding pocket of the insulin receptor consist of Val1173, Leu1219 and the aliphatic portions of Asn1215 and Glu1216 side chains (Fig. 2A). The P+3 pocket is created by residues Leu1171, Val1173, Met1176, Leu1181 and Leu1219 (13). The corresponding P+1 binding pocket residues of RETK are Ile913, Val915 and the aliphatic portion of the side chain of Glu958; however the corresponding residue to Asn1215 of IRK is a proline in RETK (Pro957) (Fig. 2A). The P+3 pocket of RETK comprise of residues Ile913, Val915, Met918, Leu923 and Phe961 (Fig. 2A).

In activated IRK, the side chain of Met1176 forms hydrophobic bonds with residues Leu1171, Val1173 and Phe1186. In receptor tyrosine kinases, this methionine is highly

conserved, whilst in cytosolic tyrosine kinases like SRC, ABL and LCK this residue is a threonine (12). In the active RETK model, the side chain of the corresponding Met918 forms hydrophobic contacts with residues Ile913, Val915 and with Tyr928 (Fig. 2B). The conformational change of the activation loop from the inactive to the active state changes the packing around this methionine residue. For example, in the inactive RETK model, the activation loop residue Ile913 is substantially displaced and does no interact with Met918. Mutation of Met918 to the smaller hydrophilic threonine, the packing around the P+1 and P+3 binding pocket, in both the inactive and the activated state, will be substantially altered. The threonine is almost completely buried, with the hydrogen of the hydroxyl gamma bound to a backbone oxygen atom (Fig. 2B). This threonine will not contribute to direct substrate binding, however due to its smaller size it allows the neighbouring residues to readjust. This probably will result in an increased P+3 binding pocket which can change the overall shape of the substrate binding sites resulting in a change of substrate specificity. Additionally, the change of packing around Ile913 might also cause an increase in constitutive RETK activity. Replacing Met918 for a smaller threonine and subsequent change of packing around Ile913 possibly permits the activation loop to change its conformation more easily from the inactive to the active conformation, thereby shifting the equilibrium between inactive and active state of RETK (Fig. 2B).

The other activating mutation of RETK described here is A883F. Although Ala883 is not highly conserved (Fig. 2A), many tyrosine kinases have a preference for relatively small hydrophilic or charged residues (Ser, Thr, Asp, Asn) or small hydrophobic residues (Gly, Ala) at this position. Residue Ala883 is located at the opposite site of the peptide binding site, in the loop connecting β-strand 7 with β-strand 8 (Fig. 2A, C). These two β-strands connect the catalytic-loop residues with the activation loop residues. Even though this Ala883 is not in direct contact with important catalytic features of RET, mutation to Phenylalanine could indirectly result in increased catalytic efficacy. The side chain of phenylalanine makes contact with Glu884 and Lys889 (Fig. 2C); this lysine is located just three residues N-terminal from the conserved DFG motive at the beginning of the activation loop (Fig. 2A). The phenyl alanine side chain is also in contact with the side chain of Glu805, located just proximal of Tyr806 and with the main chain oxygen atom of Tyr806. This Tyr806 is a known autophosphorylation site of RETK (4)**.** Modelling this A883F mutation did not reveal major

A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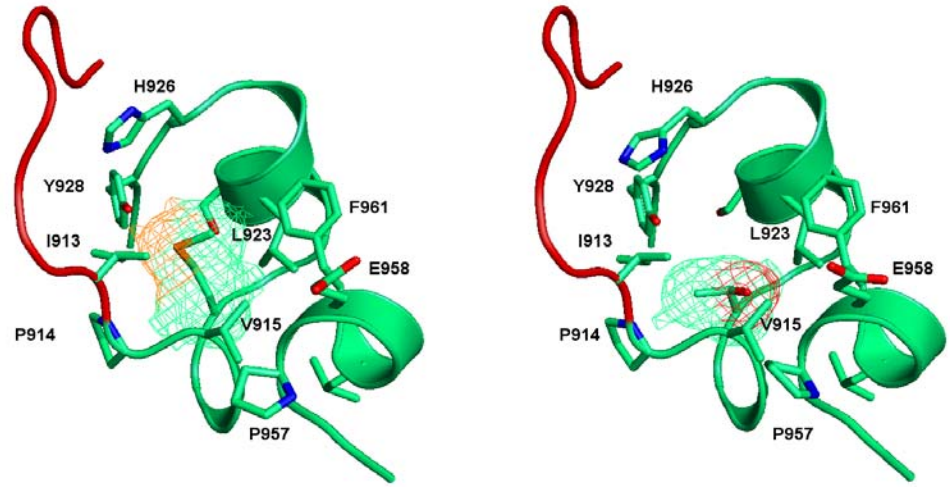
FGR1_HUMAN L V S C A Y Q V A R G M E Y L A S K K C I H R D L A A R N V L V T E D N V M K I A D F G L A R D I H H I D Y Y 654
IG1R_HUMAN M I Q M A G E I A D G M A Y L N A N K F V H R D L A A R N C M V A E D F T V K I G D F G M T R D I Y E T D Y Y 116
RET_HUMAN V K R S Q G R I P V K W M A I E S L F D H I Y T T Q S D V W S F G V L L W E I V T L G G N P Y P G I P P E R L 960
FGR1_HUMAN K K T T N G R L P V K W M A P E A L F D R I Y T H Q S D V W S F G V L L W E I F T L G G S P Y P G V P V E E L 709
IG1R_HUMAN R K G G K G L L P V R W M S P E S L K D G V F T T Y S D V W S F G V V L W E I A T L A E Q P Y Q G L S N E Q V 122

B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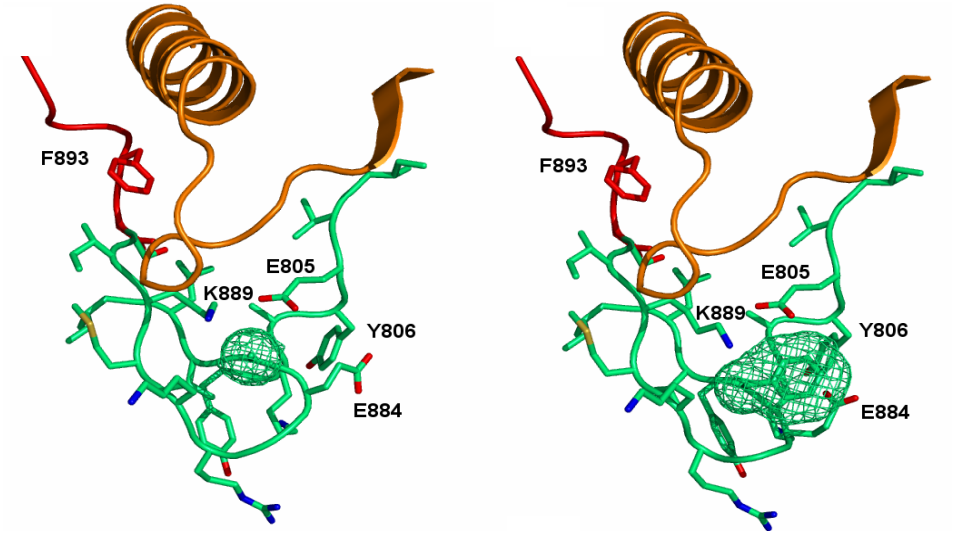
F893
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Figure 2

Amino acid sequence alignment and predicted secondary structure of the c-terminal tyrosine kinase domain of RET and structural model of RET affected by MEN2B mutations M918T and A883F.

A) ClustalW amino acid sequence alignment of RET with the human RTK homologues FGR1 and IG1R. Legend: white and black boxes are secondary structure elements, α helices and β strands, respectively in FGR1. White-grey bar: catalytic loop; dark-grey bar: activating loop; black bar (*): P+1 loop core. Filled black squares represent identical amino acid and red filled squares represent mutated amino acids. The ruler shows the sequence numbering of RET.

B) Structural model analysis of activating mutation M918T in P+1 and P+3 peptide binding pocket. The wild type RETK (left panel) and M918T RETK (right panel) models are depicted. Residues forming the P+1 and P+3 peptide binding pocket in the activated state are shown. Represented in mesh is residue 918 and C-terminal part of activation loop is colored in orange. Active loop is colored in red.

C) Structural model analysis of activating mutation A883F located between N-terminal lobe and C-terminal lobe. The wild type RETK (left panel) and A883F RETK (right panel) models are depicted. Represented in mesh is residue 883. Selected residues interacting with residue 883 are numbered, in addition residue F893 of the DFG motive is shown as reference. Activation loop is colored red. The αC helix, β4-strand and the interconnecting loop, located in the N-terminal lobe, are colored orange .

structural disturbances upon substitution of a small residue with a more bulky one. Even so, small structural adjustments will probably happen in order to accommodate this more bulky residue. Adjustments of the glutamic side chain and the main chain oxygen atom of Tyr806 can result in a small change in backbone configuration, which can result in altered accessibility of Tyr806 (Fig. 2C). The same can be proposed for the interaction with Lys889; a small readjustment of backbone configuration can cause a change of the local configuration near the DFG motive and cause a shift in equilibrium of the activation loop from an inhibited state to the active.

In conclusion, combining functional (luciferase reporter assays and Western blotting analysis) and the structural modelling studies, we have shown: i) specific activation of the ERK1/2 and STAT3 pathways by specific MEN2 disease phenotype associated RET mutants. Furthermore we showed that v-SRC can phosphorylate RET *in trans*, and this phosphorylation is more accentuated for those mutations targeting the P+1 loop in the kinase domain of RET, suggesting a novel oncogenic mechanism of RET activation for RETS891A, RETA883F and RETM918T mutants. ii) predicted the structural changes caused by MEN2B

mutants, RETA883F and RETM918T, and thereby their possible oncogenic mechanism of activation.

All together, we have brought new insights into the signalling properties associated with specific MEN2-RET oncoproteins in order to connect them with their associated disease-phenotypes.

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Chapter 5

Re: Inhibition of MTC cell proliferation and RET phosphorylation by tyrosine kinase inhibitors

Surgery, 2004, 132(6): 960-967

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Dear Sir,

An important target for cancer research is the search for efficient anti-cancer drugs. Recently, tyrosine kinase inhibitors, in particular Gleevec, also known as Glivec, STI1571 and CGP57148B, have been used very successful in the clinical management of chronic myelogenous leukemias (CMLs) and in gastrointestinal stromal tumours (GISTs). The targets of Gleevec in these cancers are the BCR-ABL and C-KIT respectively, both receptor tyrosine kinases. The success of Gleevec prompted many groups to investigate Gleevec in relation to other tumours expressing receptor tyrosine kinases similar to BCR-ABL or C-KIT. An obvious target is RET, a tyrosine kinase receptor often mutated in medullary thyroid carcinoma (MTC) and pheochromocytomas. In the December 2002 issue of this journal Cohen, Hussain and Moley describe the use of Gleevec to inhibit RET activity in a cell line derived from an MTC (ref 1.). They report that Gleevec inhibits proliferation of this MTC cell line and that Gleevec also inhibits RET tyrosine phosphorylation. The paper raises several questions that we would like to address in this letter.

The first concerns the interpretation of figure 1, which shows the effect of Gleevec on the tyrosine phosphorylation status of the RET receptor. The authors conclude from the upper panel of the figure that loss of tyrosine kinase activity correlates with an increase of the concentration of Gleevec. Indeed, the western shows a Gleevec concentration-dependent decrease in tyrosine-phosphorylated RET. However, when the same blot is reprobed with a RET antibody, this amount also decreases with increasing concentrations of Gleevec. Therefore, a more correct conclusion would be that less RET is immunoprecipitated from cells, treated with increasing Gleevec concentrations, and hence less tyrosine-phosphorylated RET is detected. Without a blot analysis of total cell lysates prior to immunoprecipitation, it is impossible to conclude how Gleevec treatment results in reduced RET protein levels.

A second question concerns the fairly high concentration of Gleevec used for the cell proliferation studies. The data would become more convincing if the authors would have tested these concentrations on a (several) control cell lines to exclude cytotoxic effects.

As presented in figure 2, both geldanamycin and genistein seem to inhibit RET phosphorylation. For geldanamycin we see, however, that when the same blot is reprobed with a RET antibody, the mature form of the receptor (170kD) decreases with the concentration of the inhibitor, suggesting an effect on the maturation of the

RET protein instead of an inhibition of the catalytic activity of the receptor. In the right panel of figure 2, we can compare it with the effect of genistein, a non-specific tyrosine kinase inhibitor, which shows the results we expect.
In conclusion, we think that RET is indeed a promising target for Gleevec inhibition. The proposed outcome of the study by Cohen and co-workers, however, in our view needs further analysis and experiments before it can be claimed that Gleevec is useful for the treatment of MTC through inhibition of RET activity.

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Chapter 6

Cellular effects of Imatinib on Medullary Thyroid cancer cells harboring Multiple Endocrine Neoplasia type 2A and 2B associated RET-mutations

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ABSTRACT

Background. Activating mutations in the RET-gene, which encodes a tyrosine kinase receptor, often cause medullary thyroid carcinoma (MTC). Surgical resection is the only curative treatment; no effective systemic treatment is available. We evaluated imatinib, a tyrosine kinase inhibitor currently used to treat chronic myelogenous leukemia (CML) and gastrointestinal stromal tumours (GIST) as a potential drug for systemic treatment of MTC, in two MTC derived cell lines expressing Multiple Endocrine Neoplasia associated mutant RET-receptors.

Methods. RET-expression and Y1062-phosphorylation was determined using western blot analysis and quantitative PCR. Effects on cell proliferation were determined by a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay and we used FACS analysis using annexin V/propidium iodide staining to study imatinib-induced cell cycle arrest, apoptosis and cell death.

Results. Imatinib inhibited RET Y1062-phosphorylation in a dose-dependent manner after 1.5 hours of exposure. After 16 hours both RET Y1062-phosphorylation and expression levels were affected. Dose-dependent decreases in cell proliferation of both cell lines after exposure to imatinib with IC_{50} concentrations of 23 ± 2 µM and 25 ± 4 µM were seen. These IC_{50} values are high compared to those for CML and GIST. We further could show that imatinib induced cell cycle arrest, apoptotic and non-apoptotic cell death.

Conclusions. Imatinib inhibits RET-mediated MTC cell growth affecting RET-expression levels in vitro in a dose-dependent manner. The concentration of imatinib necessary to inhibit RET in vitro makes it however yet impossible to conclude that imatinib monotherapy will be a good option for systemic therapy of MTC.

INTRODUCTION

Surgery is at this moment the only adequate curative intended treatment in medullary thyroid carcinoma (MTC) consisting of a total thyroidectomy with at least a central compartment dissection (1, 2). Clearance of all neoplastic tissue in the neck may improve disease-free survival with normalization of calcitonin levels. Unfortunately, more than 50% of the patients will have persistent or recurrent disease after initial surgery and are prone to develop distant metastases for which there is no adequate systemic treatment available (3-6) .The clinical presentation of MTC depends on the presence of specific mutations in the *RET*-gene (*RE*arranged during *T*ransfection), located on 10q11. The *RET*-gene encodes a receptor tyrosine kinase with an extracellular ligand binding domain (cysteine rich domain), a single transmembrane domain and an intracellular domain containing the catalytic tyrosine kinase domain (7). Oncogenic activation of receptor tyrosine kinases can occur by chromosomal translocation (as found in papillary thyroid carcinomas) and by single point mutations. Mutations in the extracellular cysteine rich domain of the RET-protein are encountered in patients having the cancer syndrome multiple endocrine neoplasia type 2A (MEN2A) and familial MTC (FMTC), while mutations in the tyrosine kinase domain are found in patients with FMTC and MEN2B (8-10). In MEN2A, the most frequently found mutation is C634R whereas in MEN2B, the most frequently observed activating mutation in *RET* is M918T (10). This mutation is also found in ~40% of sporadic MTC cases (10,11). These mutations and translocations lead to ligand-independent RET-autophosphorylation, resulting in a constitutively active RET-receptor. In the hereditary form of MTC, *RET*-mutations are always observed and RET-expression is characteristic for both the sporadic and hereditary type of MTC.

Small molecule drugs that can selectively inhibit tyrosine kinases have proven to be useful in the systemic treatment of a number of neoplastic diseases. Recently the selective tyrosine kinase inhibitor 571 (STI571, imatinib, Glivec, Gleevec, CGP57148B; Novartis Pharmaceuticals) has been developed. It belongs to the 2-phenylaminopyrimidine class and it proved to target BCR-ABL, platelet derived growth factor receptor (PDGFR) (12, 13). and c-kit receptor tyrosine kinases (14,15). Currently, imatinib is clinically used to treat chronic myelogenous leukemia (CML)

and gastrointestinal stromal tumors (GIST) as well as dermatofibrosarcoma protuberans (16-21).

Because both c-kit and RET belong to the same subfamily of tyrosine kinase receptors we investigated whether RET tyrosine kinase (RET-TK) can be inhibited by imatinib as well. Dose-dependent growth inhibition induced by imatinib in the human MTC cell line TT has been reported (22). However, others could not reproduce these data (23). Furthermore, it remains unclear whether the antiproliferative effect of imatinib is caused by inhibition of RET-autophosphorylation, or by induction of apoptosis, or through other mechanisms and several questions have been raised concerning the relatively high concentrations of imatinib used (23, 24). In addition, only one MTC derived cell-line has been used in previous studies.

Aims of this study were: [1] to investigate whether imatinib can inhibit the activity of oncogenic RET mutants *in vitro* at clinical achievable serum levels, [2] to determine whether imatinib induces growth arrest and/or cell death in two MTC cell lines; TT a cell line reported to be derived from a sporadic MTC carrying a C634W RET mutation (25, 26) and MZ-CRC-1, a cell line derived from a malignant pleural effusion from a patient with metastatic sporadic MTC carrying a M918T mutation (26, 27) and [3] to determine whether there is specificity of imatinib for particular *RET*-mutations associated with MEN2A and MEN2B.

MATERIAL AND METHODS

The human MTC cell line MZ-CRC-1 was a gift of A. Knuth, the human MTC cell line TT and the human hepatocellular carcinoma cell line HepG2 were obtained from the American Type Culture Collection, Manassas VA. RPMI-1640 medium, Dulbecco's Modified Eagle Medium (DMEM), trypsin-EDTA, L-glutamine and penicillin-streptomycin were purchased from Gibco (Invitrogen corp. Breda, the Netherlands) and Fetal Calf Serum from Integro BV (Zaandam, the Netherlands). Antibodies against RET (H300), PY-RET (Y1062) and actin (C4) were used in dilutions of 1:1000, 1:250 and 1:10000, respectively. Anti-rabbit IgG-HRPO peroxidase conjugated antibodies were used in dilutions of 1:2000. All antibodies were purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA except anti-actin, which was obtained from ICN Biomedicals (Aurora, OH, USA). Imatinib was kindly provided by Novartis Pharma AG (Basel, Switzerland) and dissolved in dimethyl sulfoxide (DMSO). MG132 was purchased from Calbiochem (Merck Biosciences, Nottingham, UK) and dissolved in DMSO.

Cell culture

MZ-CRC-1 cells were cultured in DMEM supplemented with 10% (v/v) FCS, 100 U/mL penicillin, 100 µg/mL streptomycin and 2mM L-glutamine. TT cells were cultured in RPMI-1640 supplemented with 15% (v/v) FCS, 100 U/mL penicillin and 100 µg/mL streptomycin. HepG2 cells were maintained in DMEM supplemented with 10% (v/v) FCS, 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were maintained under a fully humidified atmosphere of 95% air and 5% CO_2 at 37 ˚C.

Western blot analysis

MZ-CRC-1 cells and TT cells were grown at a density of $5x10^5$ cells/well for 48 hours and treated with different concentrations of imatinib for 90 minutes. Cells were washed with ice-cold phosphate buffered saline (PBS) and lysed with 100 µL of lysis buffer (20 mM HEPES pH 7.4, 2 mM EGTA, 1 mM DTT, 1 mM Na_2VO_3 (ortho), 1% Triton X100, 10% glycerol, 10 µg/mL leupeptin, 30 µg/mL aprotinin and 0.4 mM PMSF). Protein concentration was determined according to the Bradford method prior to SDS-polyacrylamide gel electrophoresis (28) Forty µg of total cell lysates were western blotted. Cell lysates were boiled in Laemmli buffer (4% SDS, 250 mM Tris-Cl pH 6.8, 20% glycerol, 0,01% bromphenol blue and 10% 2-mercaptoethanol) before

loading. Then they were separated on 12% SDS-polyacrylamide gels and electro blotted in Tris-glycine buffer (39 mM glycine, 48 mM Tris-Cl, 0.037% SDS, 20% methanol) onto 0.20 μM nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were incubated in blocking solution (5% nonfat-milk in TBS-T (25 mM Tris-Cl pH 8.0, 137 mM NaCl, 5 mM KCl, 0.05% Tween)) for 1 hour at 22 ˚C, followed by incubation with anti-RET (H300), anti-PY-RET (Y1062), or anti-actin (C4) in fresh blocking solution at 4 ˚C overnight. The membranes were then washed in TBS-T and incubated with a 1:2000 dilution of the indicated HRPO-conjugated secondary antibody for 2 hours at 22 ˚C. Membranes were then washed with TBS-T. Antibody detection was performed with an enhanced chemiluminescence reaction (Pierce Chemical Company, Rockford, IL, USA) according to the manufacturer's recommendations.

RNA extraction, preparation of cDNA and quantitative PCR

TT cells were cultured for 24 hors in RPMI 1640 supplemented with 10% FCS, and subsequently treated with or without 15 μM imatinib for 16 hours. Total RNA was isolated using the RNeasy Mini Kit (Qiagen GmBH, Hilden, Germany) according to the manufacturer's protocol. The RNA concentration was determined by spectrophotometry (Nanadrop). Thereafter, 1 μg of total RNA was transcribed in first-strand cDNA using the RTG First Strand cDNA kit (Amersham Biosciences, Hertogenbosch, The Netherlands) according to the manufacturer's protocol. The cDNA synthesis was primed by the pd(N)6 Random Hexamer (Amersham). Relative changes in transcript level were determined on the Icycler (Bio-Rad) using Quantitect SYBR Green PCR Kit from Qiagen, following the manufacturer's instructions.

Primer sequences used in this study were as follows: hypoxanthine phosphoribosyltransferase (HPRT)-for: cgcccaaagggaactgatagtc; HPRT-rev: agttctgtggccatctgcttag; glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-for: cacccactcctccacctttg; GAPDH-rev: ccaccaccctgttgctgtag; RET-for: tgggaattccctcggaagaa; RET-rev: tactccacgatgaggaggagc.

The PCR efficiencies for all primers used were between 96% and 99%. Data are expressed as fold induction corrected for GAPDH and HPRT. All experiments were performed in duplicate.

Proliferation assay

To determine cell proliferation, a MTT test was employed (Roche Diagnostics, Almere, the Netherlands). Briefly, cells were seeded at a density of $1x10^5$ cells/well in 96-well microtiter plates, grown overnight and exposed to different concentrations of imatinib or 0,1% DMSO as a control. After 1, 2, 3, 4 and 5 days of incubation, respectively, 10 µL of 3-(4, 5-methylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT-reagent) was added to each well and incubated at 37 °C for 4 hours. Formazan products were solubilized with 100 µL/well of the supplied detergent buffer and the plate was incubated overnight at 37 °C. The optical density was determined at 595 nm wavelength. To determine the IC_{50} of imatinib (the concentration that causes 50% growth inhibition), a concentration range of imatinib was added to the wells (quadruplicates) and IC_{50} was determined using linear interpolation at r = 0.5. All experiments were performed in triplicate.

Cell cycle and apoptosis analysis

For fluorescence-activated cell sorting (FACS) analysis cells were cultured sub confluent and treated with 15 µM imatinib for 24, 48, 72 and 96 hours. The percentage of apoptotic cells was assessed using an annexin V staining kit (IQ Products, Groningen, the Netherlands) according to the manufacturer's recommendations. Briefly, cells were harvested, resuspended in 100 µl calcium buffer (2.6 mg/mL HEPES, 0.28 mg/mL $CaCl_2$, 8.18 mg/mL NaCl) containing 5 µL of annexin V and incubated for 20 minutes at 4 °C in the dark. Cells were washed with 5 mL calcium buffer and subsequently incubated in 300 µL calcium buffer containing 2.5 µL of propidium iodide (PI) for 10 minutes in the dark. Finally, binding of fluorescein-conjugated annexin V (apoptotic death) and PI (necrotic death) was measured by FACS (FACSCalibur, Becton Dickinson, Sunnyvale, CA, USA). For assessing cell cycle distribution, cells were harvested, washed with PBS and incubated in PI solution (0.1 µg/µL Rnase A, 0.96 mg/mL Sodium citrate-dihydrate, 0.02 µg/µL PI, 0.1 % Triton X-100) for 20 minutes at room temperature. Binding of PI was measured using FACS and cell cycle analysis was performed using ModFit LT.

Statistical analysis

Values shown are depicted as mean ± standard deviation. The relationship between continuous variables was analyzed using the Student's t-test. In all tests $p<0.05$ was considered significant.

RESULTS

Imatinib inhibits RET-autophosphorylation in MZ-CRC-1 and TT cells in a dose-dependent manner

Lysates prepared from MZ-CRC-1 and TT cells treated with imatinib for 90 minutes were western blotted and analyzed with an anti-PY-RET (Y1062) antibody. Inhibition of RET Y1062-autophosphorylation was observed in a dose-dependent manner, with complete inhibition observed at concentrations higher than 15 µM in both cell lines (Fig. 1).

To exclude that the observed reduction in RET Y1062-phosphorylation was caused by decreased RET-expression levels, the membrane was stripped and reprobed with an anti-RET primary antibody. The total amount of expressed RET did not change after exposure to imatinib for 90 minutes indicating that imatinib inhibits RET-autophosphorylation but does not affect the expression level of the RET-protein (Fig. 1).

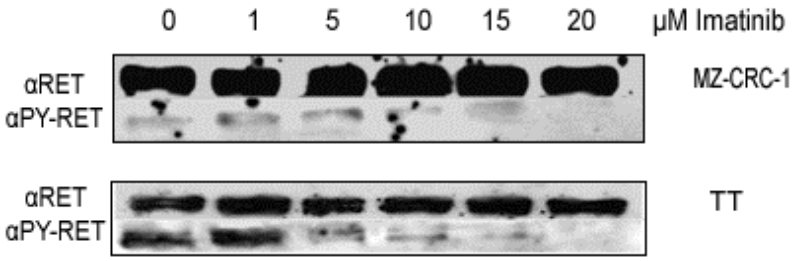


Figure 1

Dose-dependent inhibition of RET-autophosphorylation in MZ-CRC-1 and TT cells after 90 minutes exposure. MZ-CRC-1 cells and TT cells were grown at a density of $5x10^5$ cells/well for 48 hours and treated with different concentrations of imatinib for 90 minutes. Forty µg of total cell lysates were western blotted and blots were probed with anti-PY-RET (Y1062), stripped and probed with anti-RET. Loading controls were done by stripping the blot again and probing for actin.

Long-term exposure to imatinib induces RET-oncoprotein degradation

In order to investigate the effects of extended exposures to imatinib on RET-expression and phosphorylation levels in MZ-CRC-1 and TT cells; cell lysates were prepared after 16 hours of exposure to imatinib and analyzed using western blotting. Complete inhibition of RET Y1062-autophosphorylation was observed at concentrations higher than 10 µM in both cell lines (Fig. 2). Reprobing the membrane with an anti-RET antibody showed that RET-expression levels were also reduced by

long-term imatinib treatment, indicating that reduced RET Y1062-phosphorylation levels are caused by reduced RET-expression levels. In MZ-CRC-1 cells expression levels of RET-protein decreased dramatically and in TT cells RET could not be detected at concentrations of 15 and 20 µM (Fig. 2). To determine whether equal amounts of protein were loaded, blots were stripped and reprobed with an anti-actin antibody and the total amount of actin did not change after exposure to imatinib.

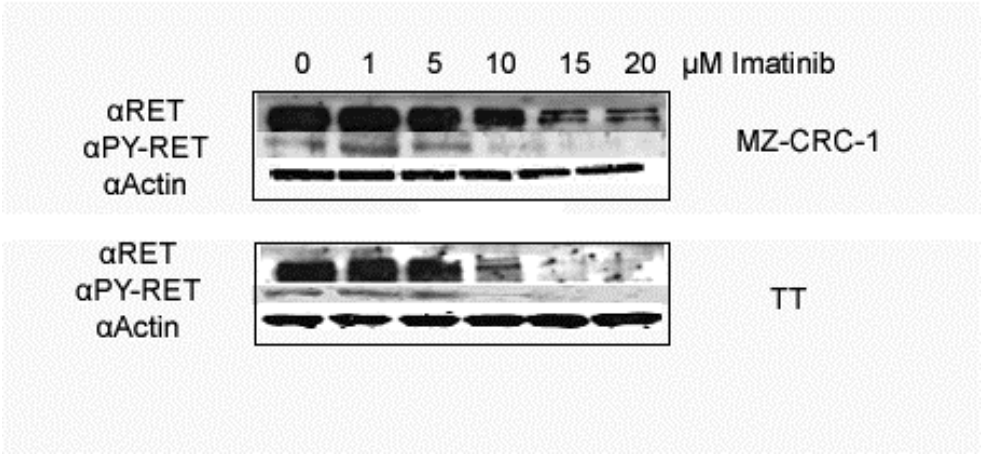


Figure 2

Imatinib inhibits both autophosphorylation and expression of RET in MZ-CRC-1 and TT cells after 16 hours exposure. MZ-CRC-1 and TT cells were grown at a density of $5x10^5$ cells/well for 48 hours and treated with different concentrations of imatinib for 16 hours. Forty µg of total cell lysates were western blotted and blots were probed with anti-PY-RET (Y1062), stripped and probed with anti-RET. Loading controls were done by stripping the blot again and probing for actin.

We used the proteasome inhibitor MG132 to determine if RET was degraded by the proteasome-ubiquitin pathway under the influence of imatinib. 10 µM MG132 was added to MZ-CRC-1 and TT cells in combination with increasing concentrations of imatinib. Cells were treated for 16 hours and whole cell lysates were western blotted and developed with an anti-RET antibody. In both cell lines, RET-expression levels were still reduced by imatinib as seen in figure 3.

Finally, we studied the effects of imatinib on RET by means of quantitative PCR (qPCR) (Fig. 4). Treatment of TT cells with 15 µM imatinib for 16 hours did not result in a decreased synthesis at the transcription level.

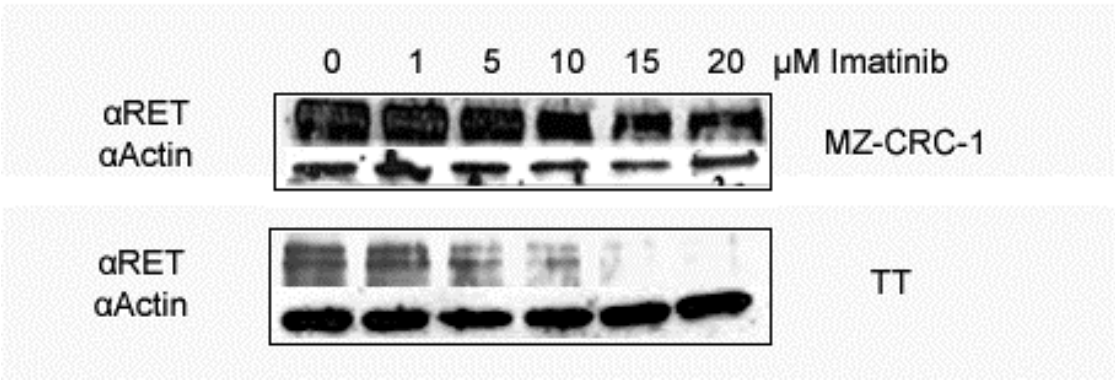


Figure 3

Imatinib treatment does not induce proteasomal RET-degradation. MZ-CRC-1 and TT cells were grown at a density of $5x10^5$ cells/well for 48 hours and treated with different concentrations of imatinib and 10 µM of the proteasome inhibitor MG132 for 16 hours. Forty µg of total cell lysates were western blotted and blots were probed with anti-RET, stripped and probed with anti-actin as a loading control.

MG132 did not have any influence on RET-expression levels and transcription was not altered by imatinib. Therefore we conclude that imatinib does not decrease RET-expression by enhancing protein degradation through the proteasome-ubiquitin pathway or inhibition of transcription.

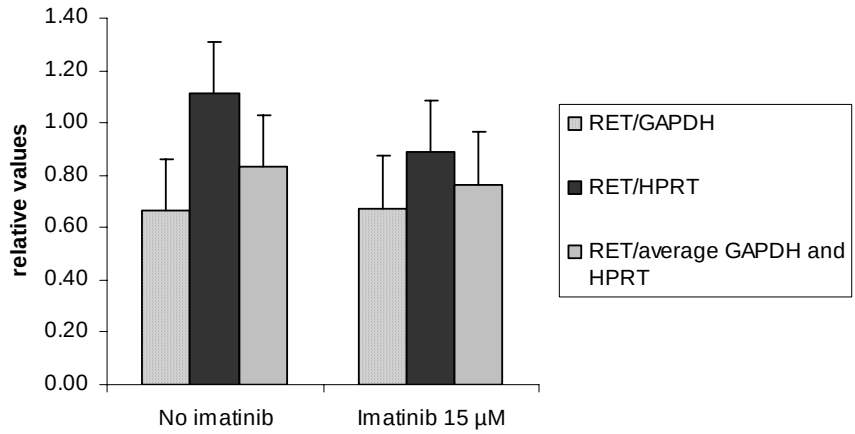


Figure 4

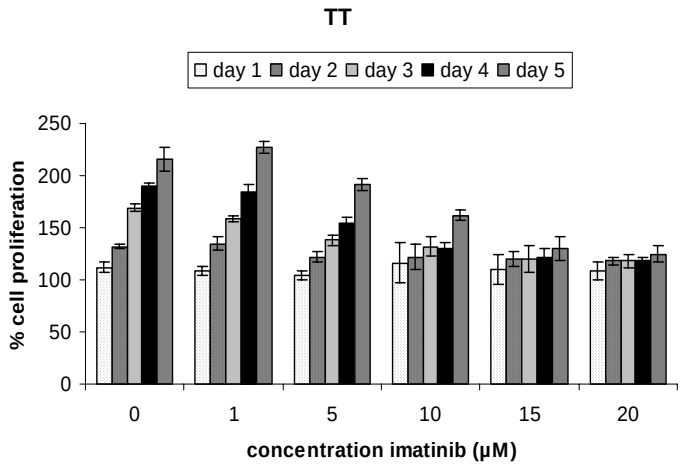
Long-term treatment with imatinib does not decrease RET-transcription. TT cells were grown in RPMI 15% FCS for 48 hours and subsequently treated with 15 µM imatinib or only RPMI 15% FCS for 16 hours. RET mRNA was quantified using quantitative PCR. Data are expressed as fold induction corrected for hypoxanthine phosphoribosyltransferase (HPRT) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and obtained from two independent experiments.

Imatinib inhibits proliferation of MZ-CRC-1 and TT cells

The effect of imatinib on cell growth of MTC derived cell lines was measured by means of a MTT assay. MZ-CRC-1 and TT cells were incubated with increasing concentrations of imatinib for up to five days (Fig. 5). Dose-dependent decrease in cell proliferation in both MZ-CRC-1 and TT cells was observed. In contrast, in HepG2 cells that do not express RET (data not shown), imatinib had no effect on the proliferation in concentrations lower than 20 µM. To determine the IC_{50} of imatinib in MTC cell lines, MZ-CRC-1 and TT cells were grown in normal FCS containing medium and exposed to increasing concentrations of imatinib (Fig. 6). The concentrations of imatinib required inhibiting cell growth by 50% were 23 ± 2 µM for MZ-CRC-1 cells and 25 ± 4 µM for TT cells.

Imatinib promotes cell cycle arrest and apoptosis

We evaluated the contribution of cytotoxic and cytostatic components to imatinib-induced growth inhibition. First, simultaneous annexin V/ PI staining was employed to determine whether cell death occurred and to clarify the nature of cell death in the treated cultures. FACS cell cycle analysis showed that treatment with imatinib increased apoptotic cell death after 3 days of treatment (Fig. 7A). The fraction of apoptotic cells is, however, relatively small. Second, we determined the distribution over the different cell cycle phases of MZ-CRC-1 cells treated with imatinib. For these experiments only MZ-CRC-1 cells were used as these proved to be diploid (data



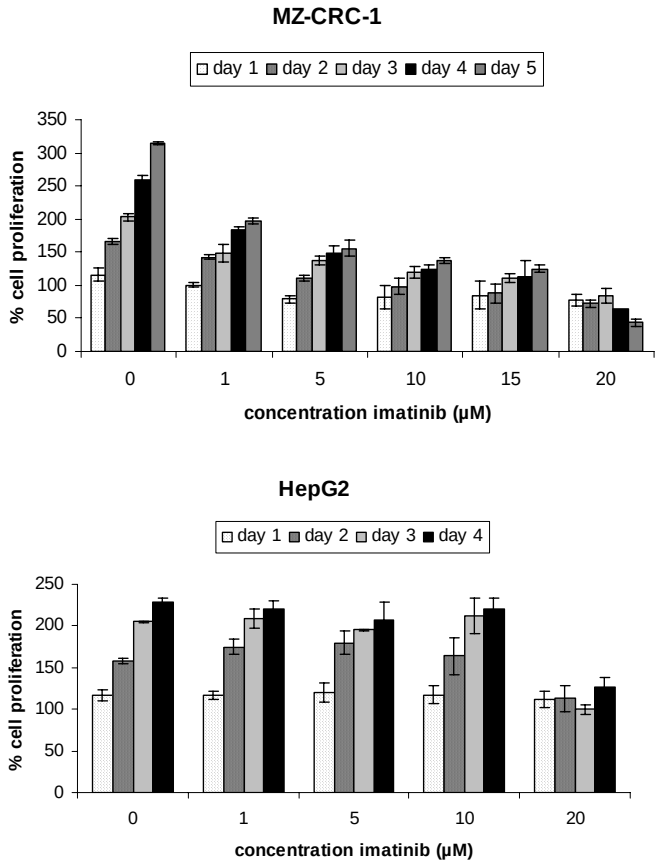


Figure 5

Imatinib inhibits proliferation of MZ-CRC-1 and TT cells in a dose-dependent manner. MZ-CRC-1 cells were seeded in DMEM 10% FCS [A] and TT cells were seeded in RPMI 15% FCS [B] at a density of $1x10^5$ cells/well and treated with different concentrations imatinib. HepG2 cells were seeded in DMEM 10% FCS [C] in a density of 5000 cells/well and served as a negative control. Cell proliferation was measured on subsequent days by 3-(4, 5-methylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide assay. Results presented here are representative of three independent experiments and are presented as mean ± SEM.

not shown) in contrast to TT cells which were described as aneuploid (http://www.biotech.ist.unige.it/cldb/totcl4901.html). As shown in figure 7B, imatinib treatment increased the percentage of MZ-CRC-1 cells in the G_0/G_1-phase of the cell cycle. This increase of cells in the G_0/G_1-phase is accompanied with a reduced number of cells in the S-phase, supporting the observations obtained with the MTT assay. The analysis did reveal considerable significant ($p<0.001$ for increase in S-phase and $p=0.003$ for changes in G_0/G_1-phase) changes in the cell cycle distribution pattern after 24 hours of incubation when compared to untreated cells. In conclusion, these data show that the main mechanism of imatinib-induced growth inhibition *in*

vitro is through cell cycle arrest in the G_0/G_1-phase, although induction of apoptosis plays a (small) role as well.

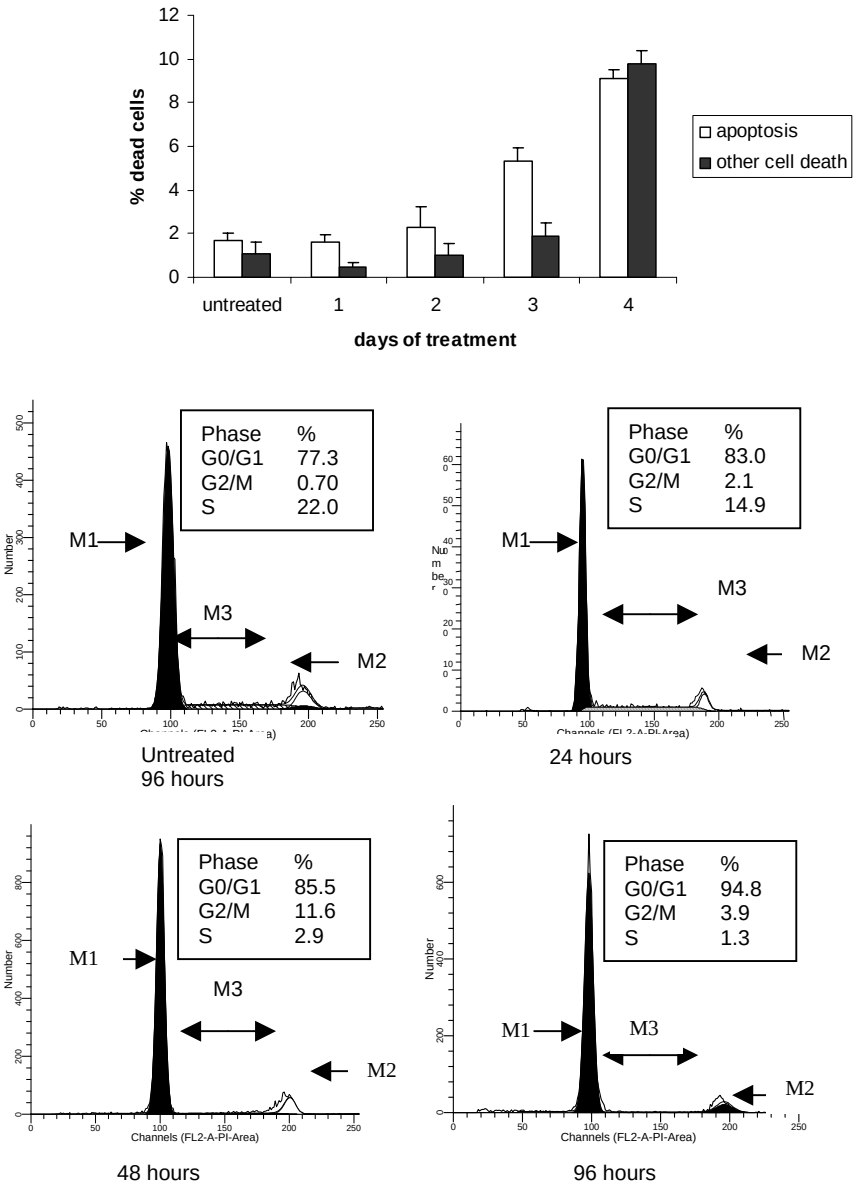


Figure 7

Imatinib promotes cell cycle arrest and apoptosis. MZ-CRC-1 cells were treated with 20 µM imatinib for 0, 24, 48, 72 and 96 hours. [A] Annexin V/PI staining revealed (relatively small) increasing percentages of apoptotic cells with increasing time of imatinib incubation. [B] Cell cycle analysis showed increased percentages of cells in G_0/G_1-phase upon longer treatment with imatinib. Furthermore, the percentage of cells in S-phasedeclined dramatically upon longer treatment with imatinib. M1 represents G_0/G_1, M2 represents G_2/M and M3 represents S-phase

DISCUSSION

The increasing knowledge of the critical signalling pathways responsible for the growth of malignancies has lead to the development of specific inhibitors of these pathways as new tools in cancer therapy. Treatment of patients with CML, GIST and dermatofibrosarcoma protuberans with imatinib is an example of the positive clinical effects of the increasing knowledge on cancer-specific signal transduction inhibition and the use of specific signal transduction inhibitors (16-21).

A tumor for which no systematic treatment is yet available is MTC. MTC is a malignancy that is caused by mutations in the *RET*-gene, which result in a constitutive active receptor tyrosine kinase (RET-TK). Recent reports have demonstrated that inhibition of constitutive active RET-TK has cytostatic or cytotoxic effects on MTC cells (22, 29-32). As the tyrosine kinase domain of RET shows high homology with that of the tyrosine kinases which can be inhibited by imatinib, we now tested the effects of imatinib on RET.

Our analyses demonstrate that imatinib indeed has a dose-dependent inhibitory effect on cell proliferation of both MZ-CRC-1 and TT cell lines, the only two available MTC cell lines. In addition imatinib does inhibit RET specifically since RET Y1062-autophosphorylation of both cell lines is selectively inhibited after 90 minutes of exposure to imatinib whereas RET-expression levels are not affected. As reduction of phosphorylation of RET was seen in both cell lines, imatinib seems not to be selective for the particular *RET*-mutations present in these cell lines (C634W and M918T).

Skinner *et al*. (23) recently reported no significant inhibition of TT cell proliferation at 10 µM of imatinib. In the present study, proliferation of both MZ-CRC-1 and TT cells is inhibited by 10 µM of imatinib and similar results were obtained by Cohen *et al*. (22). Possibly these discrepancies are caused by differences in experimental procedures employed by the different research groups. Another possibility could be that imatinib is not as effective after more than five days and that the effect is reversible.

We observed that in concentrations higher than 20 µM MZ-CRC-1 and TT cells stop proliferating. This phenomenon was also observed in cell lines that are not dependent on constitutive tyrosine kinase activity, such as HepG2 (Fig. 1). Probably these effects of imatinib on cell growth are due to general cytotoxicity.

Although we proved that RET is a direct target of imatinib we also have reason to believe that this inhibitor has other targets in TT and MZ-CRC-1. This was concluded

since we observed clear differences in concentration necessary to inhibit RET-phosphorylation compared to the concentration needed to inhibit cell proliferation. The concentration of imatinib that is able to decrease autophosphorylation and RET-content was above 10 and 15 µM, respectively. However, cell proliferation was already inhibited with 1 and 5 µM imatinib. This difference suggests that cell proliferation in MTC is probably not entirely dependent on RET-TK activity implying that imatinib acts on other cellular pathways as well.

When MTC cells are exposed to imatinib for a longer period, not only reduced Y1062-phosphorylation but also reduced levels of RET-protein were observed. This might indicate that the observed reduction in Y1062-phosphorylation after 16 hours of treatment is a consequence of reduced RET-expression levels. The studies we performed with the proteasome inhibitor MG132 show that RET-protein is not subjected to proteasomal degradation. Carniti *et al*. (32) demonstrated that PP1 inhibitors induce degradation of RET-oncoproteins through proteasomal targeting. Imatinib, however, induces RET-degradation through other means. Most likely it interferes at translation level or through proteolysis. However, the mechanism by which the inhibition of RET-phosphorylation could give rise to such a drastic effect on RET-expression levels is yet unknown. A possible link might exist between RET-activation status and RET-expression.

The main mechanism of imatinib-induced growth inhibition is that of cell cycle arrest. In cell cycle analysis we observed that imatinib induced a change in the distribution of cells over different cell cycle phases, primarily a G_0/G_1 arrest (Fig. 6B). Induction of apoptosis plays a (small) role as well. It should be noted that the population of MZ-CRC-1 and TT cells decreases after five days of exposure to imatinib (data not shown). It could be that non-apoptotic pathways play a role in cell death as well since after four days of treatment with imatinib only around 9% of cells were apoptotic. It should be noted that in these experiments cells have been used that grow in culture for many years. It is possible that primary MTCs respond differently to imatinib.

The proliferation data show dose-dependent inhibition of cell growth by imatinib with IC_{50} of 23 ± 2 and 25 ± 4 µM in MZ-CRC-1 and TT cells, respectively. These concentrations are relatively high compared to the concentrations sufficient for the inhibition of BCR-ABL and C-KIT in other cancers (Fig. 8) (33-42). Plasma concentrations of imatinib inducing hematological and cytogenetic responses in patients with CML were in the range of 0.17-5.68 µM. These patients were treated

with 25-600 mg imatinib per day (16). The maximal achievable plasma levels of imatinib in patients are no higher than 6.78 µM at maximal administered doses of 600 mg daily. Higher doses of imatinib in clinical practice are difficult to achieve because of adverse effects that are hard to tolerate for the patients (42). Moreover, the lethal effects of imatinib on BCR-ABL expressing K562 cells are only observed when greater than 50% of the cellular BCR-ABL kinase activity is inhibited (13). This suggests that a clinically relevant dose of imatinib needs to inhibit at least 50% of the target kinase activity. The IC_{50} of imatinib for RET is around four times higher than the clinically relevant plasma concentrations. Therefore, the plasma concentrations that can be achieved are not likely to inhibit RET-activity enough to be beneficial in patients with MTC although MTC can behave different in an *in vivo* model and plasma levels of imatinib do vary to a great extent among patients.

Specific inhibition of the RET-TK is a very attractive treatment option for patients with MTC. Recently the kinase inhibitors ZD6474 (29), CEP-701 and CEP-751(30) and PP1(31,32) have been shown to inhibit RET-TK at clinically achievable levels. ZD6474, an inhibitor of the vascular endothelial growth factor (VEGF) receptor-2, has a reported IC_{50} of 100 nM for RET. The indolocarbazole compounds CEP-701 and CEP-751, inhibitors of the Trk receptor tyrosine kinases, have also been shown to directly inhibit RET at nanomolar levels. PP1 shows similar IC_{50} (80 nM) for RET, but inhibits other kinases at similar or lower concentrations than RET as well (32). None of these agents, however, have been used in large-scale placebo controlled trials in humans and side effects are not yet known. Furthermore, in MTC, prolonged administration of these inhibitors is probably necessary. It would be preferable to use a tyrosine kinase inhibitor specific for RET to minimize the potential side effects.

In conclusion, we have shown that imatinib inhibits MTC cell proliferation and RET Y1062-phosphorylation leading to decreased RET-expression in cell-lines harboring MEN2A and MEN2B associated *RET*-mutations. The concentration of imatinib necessary to inhibit RET *in vitro* makes it however yet impossible to conclude that imatinib monotherapy will be a good option for systemic therapy of MTC. *In vivo* studies will be necessary to make firm conclusions concerning the clinical application of this tyrosine kinase inhibitor as an effective treatment for MTC.

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Chapter 7
Discussion: mutant RET signalling and RET as a therapeutic target in MEN2

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It has become increasingly clear that receptor tyrosine kinases (RTKs) are playing a major role in cancer development. They form a sub-class of cell-surface growth factor receptors with intrinsic ligand controlled tyrosine kinase activity. RTKs regulate crucial biological processes in the cell, but when mutated, they can become constitutively activated or display altered signalling properties, i.e. they become oncoproteins. Understanding the molecular bases of cancer will not only help us to get better insights in genotype-phenotype correlations, it might also help in designing new therapeutic approaches. Indeed, RTK-based therapies have reached widespread clinical use in, for instance, breast cancer (inhibition of HER2 by Herceptin), gastrointestinal stromal tumours (inhibition of KIT by Glivec) and non-small cell lung cancer (inhibition of EGFR by Gefitinib) (1).

In this thesis we describe one such cancer-related RTK, namely RET, and the signalling pathways in which is involved. Molecular and genetic aspects have been reviewed in the Introduction. In this Discussion we review current knowledge of mutant RET signalling, our own contribution to topic and how RET may be considered and used as a therapeutic target in MEN2.

MUTANT RET SIGNALLING

Gain of function mutations of RET result in constitutive activation of the receptor and cause MEN2. Our understanding of the molecular mechanisms that connect the mutant receptors to the different clinical MEN2 subtypes is increasing.

The specific temporal adjustment of ligand-induced AKT activation appears to be dependent of co-localization of RET in lipid rafts and this control step is bypassed by mutant RETC634R associated with MEN2A, indicated as RET-MEN2A (2). Other studies suggest that mutant RET causing MEN2B, indicated as RET-MEN2B, results in a different pattern of receptor (tyrosine) phosphorylation and in altered substrate specificity (3). Liu and colleagues (3) found that compared to wild type RET (RET-WT), the RET-MEN2B mutant lacked phosphorylation at Tyr 1096, directly leading to decreased GRB2 binding. Furthermore, expression of MEN2B mutant RET-PTC2 results, in contrast to the activated wild type receptor, in tyrosine phosphorylation of a number of proteins that interact with CRK and NCK (4). Among them is one of the CRK associated proteins, namely PAXILLIN, a cytoskeleton protein. PAXILLIN is strongly phosphorylated in RET-MEN2B, but not in RET-WT-expressing cells (4).

Furthermore, RET-MEN2B-expressing cells show selective activation of JNK-1 (5). These studies demonstrate that differences in signalling properties exist between RET-WT on one hand and RET-MEN2A and RET-MEN2B on the other. Studies comparing RET-MEN2A with either RET-MEN2B or RET-FMTC have been conducted as well. Tyrosine 1096, which is only found in the long isoform of the receptor, is phosphorylated in RET-MEN2A, but not in RET-MEN2B oncoproteins (3). Moreover, the MEN2B and FMTC-associated intracellular mutants have altered substrate specificity as in contrast to the normal substrates of receptor tyrosine kinases, they recognize and phosphorylate substrates preferred by cytoplasmic tyrosine kinases, such as c-SRC and c-ABL (5, 6). Additionally, RET-MEN2B oncoproteins trigger higher levels of activated PI3K and its downstream signalling molecules. For instance, P62DOK and PKB/AKT are phosphorylated in a PI3-K dependent manner and their levels of phosphorylation are significantly higher in MEN2B than in MEN2A cell lines (7). Moreover, tyrosine phosphorylation of P62DOK results in complex formation with the RAS GTPase-activating protein (RAS-GAP) and the NCK adaptor protein. These findings suggest that high levels of activation of PI3-K and its downstream signalling molecules may be associated with the clinical phenotype of MEN2B (7). In the same line of evidence, phosphorylation of RET tyrosine 1062 is stronger in RET-MEN2B than in RET-MEN2A (8). Phosphorylated tyrosine 1062 is part of a RET multiple effector docking site that mediates recruitment of the SHC adapter. RET-MEN2B is more active than RET-MEN2A in associating with SHC and hence the MEN2B mutant specifically potentates the ability of RET to auto-phosphorylate tyrosine 1062 and consequently triggers higher activation levels of the RAS/MAPK and the PI3-K/AKT pathways, respectively. The more efficient triggering of these pathways likely contributes to the difference between the MEN2A and MEN2B phenotypes (8).

STATs are a family of latent transcription factors involved in the activation of many genes induced by cytokines and growth factors (9). STATs when aberrantly activated, contribute to cancer development (9). In particular STAT3 has been shown to be aberrantly activated in many types of cancers, such as myelomas, leukaemia, lymphomas, melanoma, prostate, ovary, head and neck carcinomas and MTCs (10).
Oncogenic transactivation of STAT3 by RET-MEN2A C634R is required for cellular transformation, and this process is mediated by the intrinsic tyrosine kinase domains

of RET, independently of JAKs and SRC (11). In contrast, the FMTC-associated mutants RETY791F and RETS891A implicate SRC and JAKs in constitutive activation of STAT3 (10). These results suggest that RET mutants, associated with different disease phenotypes, activate STAT3 through different signalling routes. It is still not completely understood how SRC and JAKs interact with the different RET mutants in order to promote aberrant activation of STAT3. In the same line, Yuang and colleagues demonstrated that RET-MEN2B was able to interact and activate STAT3 with more affinity than RET-MEN2A mutants (12). These results corroborate a previous finding (6, 13) in which RET-MEN2B mutants were shown to be specifically associated with SRC for which STAT3 is one of the most well characterised targets.

All this points towards specific signalling profiles triggered by RET mutants associated with specific MEN2-disease phenotypes. In Chapter 4 we have compared ERK1/2 and STAT3 activation by specific MEN2-RET oncoproteins. The activation levels of both pathways correlated strongly with the degree of phosphorylation of RET Tyr1062 and RET Tyr981, respectively. Moreover, levels of RET Tyr1062 and Tyr918 were up-regulated by co-expression of a constitutive active SRC (v-SRC). The fact that RET S891A, RETA883F and RETM918T were most affected suggests *in trans* phosphorylation of RET by v-SRC as a possible oncogenic mechanism of receptor activation for point mutations in the proximity of or within the P+1 loop of the catalytic domain of RET.

In this thesis we focussed on three main pathways. Our results are discussed in more detailed below.

Specific ERK1/2 signalling by distinct MEN 2-associated RET mutants

MAPK kinase (ERK1/2) signalling plays a crucial role in the regulation of cell growth and differentiation (14). Upon growth factor stimulation, adaptor proteins will connect the receptor with guanidine exchange factors and activate RAS, which in turn will initiate a protein kinase cascade involving RAF, MEK1/2, ERK1/2 and ending with the regulation of ELK-1 and P90RSK transcription factors (14).

Deregulation of these pathways by oncogenes, such as RET, is one of the hallmarks of cancer development (15). RET-MEN2 activates the RAS/ERK1/2 pathway

independent of GDNF (see for instance Chapter 3, Figure 2A). We could demonstrate differences in the degree of ERK1/2 phosphorylation, by the different RET mutations associated with the MEN2-disease phenotypes (see Chapter 4, Figure 1). We showed that RET-MEN2B M918T induced higher levels of ERK1/2 phosphorylation than with RET-MEN2A C634R and (in decreasing order) RET-MEN2B A883F, RET-FMTC S891A, RET-FMTC Y791F and RET-MEN2A C620R, respectively. These finding strongly correlate with the levels of RET tyrosine 1062 phosphorylation displayed by the different RET mutants (Figure 1, Chapter 4), with the exception of RET-FMTC S891A. This suggests that RETS891A could preferentially trigger others signalling pathways than the RAS/ERK1/2 route via tyrosine 1062. Taken together all these results suggest that a differential degree of the ERK1/2 activation by mutant RET contributes to the clinical differences observed in MEN2.

STAT3 activation by distinct MEN2-associated RET mutants

STAT3 activation by cytokine receptors (which lack intracellular kinase domains) involves members of the JAK family of kinases (9), whereas STAT3 activation by tyrosine kinases receptors seems to be mediated by the kinase domain of the receptor itself. However, this mechanism is not completely understood. In the case of RET (as shown in Chapter 2), constitutive activation of STAT3 by two FMTC mutations (RETY791F and RETS891A) was SRC and JAK1/2 dependent. For the RET-MEN2A mutation RETC634R constitutive activation of STAT3 is independent of JAKs and SRC (11). These results suggest that different mutant RET receptors can activate STAT3 via different mechanisms.

STAT3 activation is triggered by tyrosine 705 phosphorylation, which results in dimerization and nuclear translocation (9). Activation of STAT3 can be further enhanced by phosphorylation of Ser727, located in the TAD domain (c-terminal transcriptional activator domain). In Chapter 3, we showed that RET was able to induce STAT3 Ser727 phosphorylation via a RAS/RAF/MEK1-2/ERK1-2 pathway. Interestingly, integration of the ERK1/2 and STAT3 pathways was only achieved by oncogenic RET (RETC634R, Y791F and S891A). Wild type RET did not induce STAT3 Tyr705 phosphorylation. Thus, interplay between the STAT3 and ERK1/2 pathways by oncogenic RET may play an important role in the development of MEN2.

Phosphorylation of RET *in trans* by SRC

SRC and the SRC-tyrosine kinase family of proteins are regulatory proteins that play key roles in cell differentiation, motility, proliferation and survival (16). The SRC-kinase family of proteins is highly regulated and interacts with a wide variety of molecules, such as growth factor receptors, integrin receptors, G-protein couple receptors, cytokine receptors and steroid hormone receptors (16). Several molecules have been shown to be downstream substrates of SRC, such as P85 (the regulatory subunit of PI3-K), RAS-GAP, SHC, PLC-γ, several integrin signalling proteins and FAK (16). Interestingly, all these molecules have also been shown to be downstream substrates of the RET receptor (6, 17). Furthermore, during signalling an interaction between SRC and wild type RET via Tyr981 was demonstrated (18) albeit under the specific condition of an overexpression of both wild type RET and SRC in HEK293 cells (18). In another study by Kato et al. (19), a novel SRC kinase-mediated repair mechanism of function-impaired RET mutants is reported.

In Chapter 4, we showed that the RET-MEN2B mutants (RETA883F and RET M918T) and the RET-FMTC mutant (RET S891A) affecting the intracellular part of the RET protein display higher levels of phosphorylation of tyrosine 981 (the SRC docking site) and tyrosine 1062 phosphorylation, than other MEN2-FMTC mutants. The level of tyrosine 981 phosphorylation seems to correlate with the position of the disease-associated mutations, in the sense that the highest levels of phosphorylation are found associated with mutations close to or within the P+1 loop motif.

The levels of tyrosine 981 phosphorylation (and hence SRC activation) strongly correlate with the levels of STAT3 Tyr705 phosphorylation. This finding is strengthened by the fact that STAT3 is one of the main and best-characterized SRC target molecules (13). The strong interaction between RET and SRC was further confirmed by transfecting either wild type RET or specific RET mutants in combination with a constitutively active SRC in HEK293 cells (see Figure 1, Chapter 4). In these experiments, phosphorylation levels of RET Tyr1062 and Tyr918 were up-regulated and interestingly, RET mutants RETS891A, RETA883F and RETM918T were most affected by SRC. Taken all together, these results suggest that phosphorylation *in trans* of RET by SRC is a novel mechanism of oncogenic RET activation, in particularly for those intracellular mutants targeting the P+1 loop of the kinase domain.

In summary, evidence is accumulating signalling profiles exist that are RET mutation-specific. It should be noted, that most of these studies, including ours, have been performed in artificial systems using:
i) cells derived from medullary thyroid carcinomas or ii) transient and established cell lines not always relevant to the pathobiology of RET. This and the fact that mostly specific substrates/pathways have been examined makes it likely that the present delineation of these RET mutation specific signaling profiles which underlie the different MEN2 clinical phenotypes, is far from complete.

RET AS A THERAPEUTIC TARGET

The successful inhibition of a variety of tyrosine kinases has been a recent success in the fight against cancer. Examples are Herceptin, Glivec and Gefitinib in breast cancer, gastrointestinal stromal tumours and non-small cell lung cancers, respectively (1). These findings have directed attention to RET as a possible therapeutic target in particular in MEN2 and FMTC (20) as no systematic treatment is yet available. All the crucial steps in the activation and signalling of RET, including dimerization, autophosphorylation, recruitment of adaptor proteins to various docking sites and initiation of signal-transduction cascades may be targets of specific inhibitors.

Drugs interfering with the dimerization of RET

The neutralizing, nuclease-resistant D4 aptamer is capable of binding and inhibiting wild type RET and RET-MEN2A mutant on the cell surface. The fact that monomeric RET-MEN2B was not affected suggests that D4 acts by interfering with the formation of a stable, active RET dimmers (21). The efficacy of D4 application as a therapeutic tool for RET-associated tumours needs, however, to be established.

Drugs interfering with the autophosphorylation of RET

Several RET tyrosine kinase inhibitors have been identified over the last years. Imatinib, a 2-phenylaminopyrimidine, has been shown to display inhibitory activity against RET-MEN2A and RET-MEN2B in MTC-derived cell lines and to induce RET degradation. Concentrations resulting in growth inhibition were, however, relatively high. Consequently, Imatinib will most likely not be a good candidate for systemic therapy in RET-associated tumours (22, 23). However, encouraging results

were obtained using other tyrosine kinase inhibitors such us CEP-701 and CEP-751 (20). Therefore, tyrosine kinase inhibitors might become a suitable therapy for RET-associated cancers, although as this has already been demonstrated for RET (24), the development of resistance may pose a problem in tumours treated by these agents.

Drugs interfering with the recruitment of adaptor proteins

It has been shown that SHP-1, a cytoplasmic phosphatase, can associate with mutant RET, reducing its autophosphorylation rate and consequently suppressing the growth-promoting signals of the RET-induced MAPK pathway (25, 26). This reduction in autophosphorylation is activated by somatotropin release-inhibiting factor (SRIF) (26). SHP-1 may therefore represent a molecular target for future treatment of RET-associated cancer.

Intracellular signalling

Proteins downstream of RET in different signalling pathways might be inhibited resulting in a modulation of the effects caused by the (mutant) protein. Several inhibitors that target the RAS/RAF/MEK/ERK pathway- a signalling route activated by RET (see Chapter 3), are under clinical evaluation in non-RET-associated human cancer (27). Of particular interest in RET-associated tumours is the bi-aryl urea BAY 43-9006 (sorafenib), which was initially developed as a specific inhibitor of BRAF. However, subsequent studies revealed that BAY 43-9006 also inhibits other kinases including RET (Plaza-Menacho and Hofstra, unpublished data). The simultaneous action at two levels of the same signalling pathway (RET and BRAF) in endocrine tumours may offer a perspective to avoid the development of treatment resistance.

Internalization and nuclear translocation

Targeting RET with a monoclonal antibody may be a useful therapeutic approach for neural crest cell derived cancers. Yano *et al*. have generated an antibody capable of causing internalization of RET, but its efficacy has not yet been demonstrated (28).

Biosynthesis

Gene therapeutic approaches involving the integration of new genetic material into the genome can be used to replace defective genes or to alleviate the effects of unwanted

ones by the introduction of a counteracting gene. Inhibition of oncogenic RET signalling by expression of a dominant-negative RET mutant has been investigated and reviewed by Putzer *et al* (20). Another gene therapeutic approach may be the introduction of a RET-selective ribozyme that specifically cuts mutant RET RNA and blocks RET-mediated cell growth and transformation (29).

In conclusion, the RET receptor represents a potential target for the neuroendocrine tumours observed in MEN2 in which RET dysfunction plays a crucial role. Elucidation of the signalling properties of specific RET mutants associated with the three clinical subtypes will help to uncover the molecular basis of the MEN2 disorder and thereby to design new therapeutic strategies.

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SUMMARY

The hereditary cancer syndromes Multiple Endocrine Neoplasia type 2A and 2B (MEN2A en 2B) and the related syndrome Familial Medullary Thyroid Cancer (FMTC) are caused by mutations in the *RET* oncogene. The *RET* gene encodes a transmembrane tyrosine kinase receptor which can be activated by extracellular ligands, like glial-derived neurotrophic factor (GDNF), which leads to the activation of intracellular signalling pathways (signal transduction). The research described in this thesis focused on the functional characterization of several specific MEN2-associated RET mutations in order to connect the signaling properties of the mutated receptors with their associated disease phenotypes. Furthermore, we tested the tyrosine kinase inhibitor Imatinib (Glivec) as a potential inhibitor of (oncogenic) RET, as a first step towards it use as a potential therapeutic drug in patients with MEN2.

In **chapter 1** the genetic aspects concerning *RET*, signalling by wild type RET and the *RET*-associated diseases are reviewed.

In **chapter 2** we present a study on the signalling properties of two FMTC-associated RET mutants (Y791F, S891A), both mutations affecting the intracellular RET tyrosine kinase domain. We demonstrate that these mutant receptors signal independently of GDNF and as monomeric oncoproteins. Moreover, using reporters assays and western blotting, we could show that these mutants strongly activate the STAT3 signalling pathway when compared with the wild type RET receptor. This was also confirmed in tumours from patients carrying a RETY791F germline mutation. Furthermore, by interfering with various signalling pathways, we could show that aberrant activation of STAT3 by RETY791F and RETS891A was mediated by a SRC/JAK1/JAK2-dependent mechanism. This SRC/JAK1/JAK2 dependency of FMTC-RET mutants differs from previous findings obtained with a MEN2A-RET mutant, RETC634R, which showed SRC/JAK1/JAK2 independency. In order to get insight into the structural-molecular mechanisms by which these intracellular point mutants affect the tyrosine kinase domain of RET, we modeled the tyrosine kinase domain of these FMTC RET mutant proteins. We were able to show that the amino acid substitutions probably change the structure of the activation loop and thereby change the activation loop to a more open substrate and ATP binding conformation. Nevertheless, the exact molecular

mechanisms by which SRC and JAK1 and 2 interact with oncogenic RET still needs to be investigated further.

In **chapter 3**, we demonstrate that oncogenic RET (RETC634R, RETY791F and RETS891A) can induce constitutive phosphorylation of STAT3 Ser727. RET wild type is also able to induce ligand-dependent phosphorylation on Ser727, but is unable to phosphorylate STAT3 on Tyr705. Using various strategies, we demonstrated that RET induced STAT3 Ser727 phosphorylation through a pathway involving RAS-RAF-MEK1/2-ERK1/2 was required to achieved maximal transcriptional activition by STAT3. Moreover, inhibition of ERK1/2 resulted in both, decreased phosphorylation of STAT3Ser727 and reduced proliferation of MTC-TT cells, a metastatic tumor cell line expressing RETC634W. In biopsies from patients carrying a germline RETS891A mutation, strong nuclear staining of phosphorylated ERK1/2 and Ser727 phosphorylated STAT3 was observed in the tumor tissue. These data suggest that oncogenic RET mutants strongly activate STAT3 through STAT3-Y705 (Chapter 2) and Ser727 (Chapter 3) phosphorylation, which could play an important role in the development of MTCs.

Chapter 4 Despite clear genotype-phenotype correlations in the RET-associated MEN2 cancer syndrome, the molecular mechanisms connecting the mutated receptors with their distinct clinical subtypes are far from completely understood. In chapter 4**,** we tried to find specific signalling properties for the different disease associated RET mutants by reporter assays, western analysis in combination with structural modeling studies. In this study we show that the ERK1/2, STAT3 and SRC signaling pathways are differentially activated by specific MEN2-RET oncoproteins and that the level of activation of these pathways is correlated with the degree of RET Tyr1062 phosphorylation, a tyrosine important for activation of the ERK1/2 pathway, and RET Tyr981 phosphorylation, a tyrosine involved in the activation of STAT3 and SRC. Moreover, phosphorylation levels of RET Tyr1062 and Tyr918 were upregulated by activated SRC, suggesting a direct implication of SRC in the regulation of RET activation and signaling.

In **chapter 5 and 6** we describe the use of Imatinib (Glivec), a tyrosine kinase inhibitor known to target the receptor tyrosine kinases BCR-ABL, PDGFR and c-KIT, as an

potential inhibitor against RET. Currently, Imatinib is clinically used to treat chronic myelogenous leukemia (CML) and gastrointestinal stromal tumors (GIST) as well as dermatofibrosarcoma protuberans.

We studied the effect of Imatinib on RET in two MTC derived cell lines expressing MEN 2 associated mutant RET receptors, MTC-TT and MZ-CRC-1, respectively.

We could show that Imatinib inhibits RET Tyr1062 phosphorylation in a dose-dependent manner after 1.5 hours of exposure. Interestingly, after 16 hours of exposure to Imatinib, both RET Tyr1062-phosphorylation and RET protein levels were decreased. A dose-dependent decrease in cell proliferation of both cell lines after exposure to Imatinib with IC_{50} concentrations of 23 ± 2 μM and 25 ± 4 μM was seen. These IC_{50} values for MTCs are high compared to those for CML and GIST. We further could show that Imatinib induced cell cycle arrest, apoptotic and non-apoptotic cell death. In conclusion, we showed that Imatinib inhibits RET-mediated MTC cell growth-proliferation affecting both phosphorylation and protein levels of RET in a dose-dependent manner. However, the concentration of Imatinib necessary to inhibit RET phosphorylation (5 to 10 μM) did not match the concentrations required to inhibit the proliferation of the tumor cell lines (IC_{50}=23 ± 2 μM for MTC-TT and 25 ± 4 μM for MZ-CRC-1). These suggest that other alterations are implicated in the tumorigenesis of MTCs and this makes it yet impossible to conclude that Imatinib is a good candidate for systemic therapy of MTC.

In **chapter 7** The observations presented in this thesis are discussed in view of the signalling properties of mutant RET-MEN2 proteins and the possible use of RET in designing systematic treatment for MEN2 is discussed.

NEDERLANDSE SAMENVATTING

De erfelijke kankersyndromen Multipele Endocrine Neoplasieën type 2A en B (MEN2A en 2B) en het verwante syndroom familiaire medullaire schildklier kanker (FMTC) worden veroorzaakt door mutaties in een gen genaamd *RET*. Het *RET* gen codeert voor een membraan-gebonden tyrosine kinase. Dit is een eiwit dat wordt gestimuleerd door een verbinding (met een eiwit, een zogeheten ligand) van buiten de cel, wat resulteert in het doorgegeven van een signaal (signaal-transductie) in de cel. Het onderzoek richtte zich op het functioneel karakteriseren van de mutante RET eiwitten zoals die voorkomen bij patiënten met MEN2/FMTC, om zo de functionele eigenschappen van het gemuteerde eiwit te relateren aan het ziektefenotype.
Bovendien is de tyrosine kinase remmer Imatinib (Glivec) getest als potentiële remmer van (oncogenetisch) RET, als een eerste stap om de bruikbaarheid van de remmer als therapeutisch middel voor patiënten met MEN2 te onderzoeken.

In **hoofdstuk 1** wordt de signaal-transductie van het normale RET eiwit besproken, wordt een overzicht gegeven van met *RET*-mutaties gerelateerde ziekten en wordt aandacht gegeven aan de genetische aspecten die hiermee zijn verbonden.
In **hoofdstuk 2** presenteren we een studie over de signaal-transductie van twee RET mutaties die voorkomen bij FMTC. Deze FMTC-gerelateerde RET mutaties (Y791F, S891A), zijn beide gelokaliseerd in het tyrosine kinase domein, een domein dat de activiteit van het eiwit bepaald. Aangetoond wordt dat de eiwitten waar deze mutante-genen voor coderen, onafhankelijk van een signaal van buiten, dus ligand-onafhankelijk, actief zijn. Verder wordt aangetoond dat dit gebeurt in een monomere vorm, in tegenstelling tot het “wild type” RET eiwit dat alleen actief kan worden in een dimere vorm en alleen als binding van het ligand aan RET heeft plaats gevonden. In vergelijking met “wild type” RET, bleken deze mutante eiwitten zich sterk te richten op de activatie van STAT3. Dit werd ook bevestigd in tumoren van patiënten die deze kiemlijn mutaties dragen. Bovendien bleek STAT3 activatie door deze FMTC mutaties afhankelijk te zijn van SRC en JAK1/2. Deze SRC en JAK1/2 afhankelijkheid verschilt van eerdere bevindingen met een andere specifieke MEN2 mutatie (RETC634R) die ook zorgt voor STAT3 activatie, maar dan onafhankelijk van SRC en JAK1/2. Om ook inzicht te krijgen in mogelijke veranderingen in de conformatie van deze mutante eiwitten, is het tyrosine kinase domein, waar beide mutaties zich bevinden, gemodelleerd. De ruimtelijke modellen laten zien dat de mutaties zeer waarschijnlijk structurele veranderingen teweeg brengen die leiden tot andere substraat-binding.

Activering van STAT3 vindt plaats door fosforylering van tyrosine residu 705 (Tyr705). Dit resulteert in dimerisatie en translocatie van het actieve STAT3 naar de celkern (zie **hoofdstuk 3**). In de celkern kan de transcriptie activiteit van STAT3 verder worden versterkt door fosforylering van serine residu 727 (Ser727). In hoofdstuk 3 wordt aangetoond dat gemuteerd RET (RETC634R, RETY791F and RETS891A) fosforylering van zowel STAT3 Tyr705 als STAT3Ser727 kan veroorzaken. Dit in tegenstelling tot het "wild type" RET eiwit, dat wel in staat is STAT3 op serine 727 te fosforyleren, maar dit niet kan op tyrosine 705. Verder bleek dat deze STAT3 Ser727 fosforylatie plaatsvindt via een RET-geactiveerde RAS-RAF-MEK1/2-ERK1/2-cascade. Deze *in vitro* gegevens werden gesteund door analyses van tumoren van patiënten met een kiemlijn-RET S891A mutatie. Dit suggereert dat deregulatie van de STAT3- en RAS/MAPK-signaal routes door mutant RET invloed heeft op de activatie van STAT3 en dat die activatie een belangrijke rol speelt in de ontwikkeling van MTCs.

Ondanks de duidelijke genotype-fenotype correlaties in deze kanker syndromen is het nog niet duidelijk hoe de gemuteerde receptoren leiden tot de verschillende klinische subtypes. In **hoofdstuk 4** is daarom geprobeerd specifieke eigenschappen te vinden voor de verschillende ziekte-geassocieerde RET-mutanten. In dit onderzoek bleek dat de ERK1/2-, STAT3- en SRC-signaal-transductie routes door specifieke MEN2-RET eiwitten verschillend worden geactiveerd en dat het niveau van activatie correleert met de mate van RET Tyr1062 en RET Tyr981 fosforylatie. Gefosforyleerde tyrosine 1062 is belangrijk voor de activering van de ERK1/2 route en fosforylering van tyrosine 981 is betrokken bij het activeren van STAT3 en SRC. Met betrekking tot tyrosine 981 werd verder duidelijk dat het fosforylerings-niveau het hoogst was voor de intracellulaire puntmutaties. Bovendien kon het fosforylerings-niveau van RET Tyr981 worden verhoogd door het inbrengen van een constitutief actief SRC, wat impliceert dat SRC betrokken is bij de regulatie van RET activatie en signaal-transductie.

In de **hoofdstuk 5 en 6** worden inleidende onderzoeken beschreven te aanzien van het gebruik van Imatinib (Glivec), een tyrosine kinase remmer, als mogelijk therapeutisch middel bij MEN2. Imatinib wordt reeds klinisch toegepast bij behandeling van chronische myeloide leukemie (CML) en gastrointestinale bindweefsel tumoren (GIST), alsook bij dermatofibrosarcoma protuberans. Het middel inactiveert respectievelijk BCR-ABL, PDGFR en c-KIT, alle drie receptor tyrosine kinases, net als RET.

Het effect van Imatinib op RET is bekeken in twee van MEN2 afgeleide cellijnen. Met western blotting en een serie biologische analyses kon worden aangetoond dat Imatinib RET Tyr1062 fosforylering verlaagt en wel op een dosis afhankelijke wijze. Dit werd gemeten na 1½ uur blootstellen aan de stof. Opmerkelijk is, dat als de cellen 16 uur werden blootgesteld aan Imatinib, zowel RET Tyr1062-fosforylering als de hoeveelheid RET-eiwit afnam.
Verder werd bij beide cellijnen een dosisafhankelijke afname in cel proliferatie gemeten na blootstelling aan Imatinib met een IC_{50} concentratie van 23 ± 2 μM en 25 ± 4 μM. Deze IC_{50} waarden zijn echter erg hoog vergeleken met die voor CML en GIST.
De conclusie is dat Imatinib RET-gemedieerde MTC celgroei remt en fosforylering en eiwit niveaus van RET op een dosisafhankelijke wijze beïnvloedt.

In hoofdstuk 7, de algemene discussie, wordt de signaal-transductie van de mutante RET-MEN2 eiwitten en onze bijdrage daaraan besproken, evenals het mogelijke aanpakken van deze RET mutanten bij het ontwerpen van systemische behandelingen voor MEN2.

We concluderen dat er verschillen in signaal overdracht tussen de verschillende MEN2-geassocieerde mutante RET eiwitten bestaan en dat deze verschillen waarschijnlijk een bijdrage leveren aan de klinische verschillen die geassocieerd met de verschillende MEN2 mutaties voorkomen. Bovendien kunnen we concluderen dat Imatinib RET kan remmen. Of het een bruikbaar medicijn zal blijken voor MEN patiënten is echter nog onduidelijk

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LIST OF PUBLICATIONS

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